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UNIVERSITY OF ALBERTA

ENZYME SUPPLEMENTATION AND EXOCRINE PANCREATIC SECRETIONS IN PIGS

BY

SHAOYAN LI

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ANIMAL NUTRITION

DEPARTMENT OF AGRICULTURAL, FOOD AND NUTRITIONAL SCIENCE

UNIVERSITY OF ALBERTA

EDMONTON, ALBERTA

SPRING, 1996

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DEGREE: **DOCTOR OF PHILOSOPHY**

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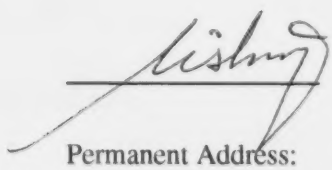

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THE DIFFERENCE BETWEEN ORDINARY

AND

EXTRAORDINARY IS THAT LITTLE EXTRA

DEDICATION

TO THE MEMORY OF MY PARENTS FOR THEIR LOVE AND EDUCATION

TO MY WIFE, AIYING CHENG, AND DAUGHTER, JULIA, FOR ALL THEIR LOVE, DEDICATION, PATIENCE AND SUPPORT

ABSTRACT

Studies were carried out to determine: (1) the effect of β-glucanase supplementation to swine diets, (2) the effect of soybean trypsin inhibitors (SBTI) on pancreatic secretions and nutrient and gross energy (GE) digestibilities, and (3) the effect of dietary crude protein (CP) content on pancreatic secretions. In Experiment 1, β-glucanase supplementation to cereal-based soybean meal diets for young pigs (6.2 to 11.2 kg) improved ($P < .05$) the apparent digestibilities of GE and CP in hulless barley-soybean meal, but not in wheat-, corn- or rye-soybean meal diets. In Experiment 2, the supplementation of β-glucanase to a hulless barley-soybean meal diet for young pigs (7.3 to 11.4 kg) also improved ($P < .05$ or $P < .01$) the ileal digestibilities of GE and the majority of the amino acids. The net disappearance of GE and ß-glucans in the large intestine was lower in the enzyme supplemented than control diet. With the exception of the fecal digestibility of GE which was improved slightly, there was no effect ($P > .05$) of β-glucanase supplementation to the wheat-soybean meal diet on the ileal and fecal digestibilities of GE and amino acids. In Experiment 3, studies were carried out to determine the response of pancreatic secretions to feeding diets containing high and low levels of SBTI in growing pigs (33.5 to 41.8 kg). Two model diets were formulated based on corn starch and Nutrisoy (a food grade defatted soy flour) and autoclaved Nutrisoy which contains a relatively high and low level of SBTI, respectively. The daily secretion volume of pancreatic juice was higher ($P < .01$) when the Nutrisoy diet was fed. However, there was no effect ($P > .05$) of diet on the total activities of chymotrypsin and trypsin in pancreatic juice. The effect of dietary SBTI contents on nutrient and GE digestibilities was determined in Experiment 4 with growing pigs (53.3 to

71.8 kg) fed corn starch-based Nutrisoy and autoclaved Nutrisoy diets. The ileal and fecal digestibilities of GE, CP and amino acids were considerably lower ($P < .01$) when the Nutrisoy compared with the autoclaved Nutrisoy diet was fed. Feeding the high SBTI diet increased the net disappearance in the large intestine of GE, CP and all amino acids measured. Finally, in Experiment 5, studies were carried out to determine the effect of dietary CP level (12 versus 24% CP from soybean meal) on pancreatic secretions in growing pigs (24.0 to 40.2 kg). Dietary CP content did not affect ($P > .05$) total protein secretion and total enzyme activities in pancreatic juice. In conclusion, ß-glucanase supplementation to a hulless barley-based diet for young pigs improved the apparent digestibilities of GE and CP. A diet high in SBTI increased ($P < .05$) the volume of secretion of pancreatic juice, but not the total enzyme activities. Ileal and fecal digestibilities of nutrients and GE were lower ($P < .05$) in pigs fed the high rather than low SBTI diet. There was no effect ($P > .05$) of dietary CP content on total protein output and enzyme secretion in pancreatic juice of growing pigs.

ACKNOWLEDGMENTS

Upon preparation of this thesis, I continually recall all the steps my supervisor, Dr. Willem C. Sauer, led me through into science. His dedicated supervision, understanding, patience, encouragement and friendship made this thesis possible. His scientific attitude and philosophies have undoubtedly changed my life, and will continue to influence me in my future career. All I want to say is one word: Thank you, Willem !

I would like to extend my sincere thanks to the members of my supervisory and examination committee, Drs. Mathison, Robinson and Sim for their encouragement and dedicated supervision. I would like to thank Drs. Chavez and Thacker for serving as external examiners, Dr. Sim for chairing the final oral examination and Dr. Basu for participating in my candidacy examination.

I would like to thank Dr. Price, the Chairman of the former Department of Animal Science, for placing the facilities at my disposal. I am very grateful to Mr. Terry Fenton for his timely technical assistance and encouragement in pursuing my Ph.D program. My sincere thanks to Margaret Micko for her friendship and help whenever I needed it.

My sincere thanks to Drs. Aherne, Hardin, Hudson, McBurney, Ozimek and Micko for their guidance and constructive advice. I am indebted to the following individuals for their help and friendship: Gary Sedgwick, Mira Fenton, Anya Nowinska, Ray Weingardt, Gavin Godby, Brian Turner, Brian Kerrigan, Paul Gregory, Steve Melnyk, Brenda Tchir, Charlane Gorsack, Clinton Lysgaard, Judy Carss and all staff members and graduate students in this Department, particularly Mike Dugan, Kelvin Lien, Ming Zhe Fan, Vince Gabert, Suxi Huang, William Caine, Mingfu Liu, to name a few, and technician Rick Allan. They have been always ready to help me and, more importantly, to give me a boost when my energy was exhausted.

Pursuit of this degree has been made possible by the encouragement of Shandong Agricultural University of my home country and by the financial support from the Natural Sciences and Engineering Research Council of Canada and the Alberta Agricultural Research Institute.

Finally, I wish to thank my wife, Aiying Cheng, for her love, patience, endurance, support and encouragement. Her hard work and timely help always sets up an example for me to go forward. She has spent twice as much time as I have on this thesis and is very much willing to let me have this degree. My daughter Julia has been a great kid and always understands that Daddy's work has been always more important than playing with her.

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LIST OF ABBREVIATIONS NOT DEFINED IN THE TEXT

ABBREVIATION	DEFINITION
ADF	Acid detergent fiber
ADG	Average daily gain
BW	Body weight
°C	Degree Celsius
CP	Crude protein (N x 6.25)
d	Day
EE	Ether extract
DM	Dry matter
DMI	Dry matter intake
FE	Feed efficiency
FI	Feed intake
GE	Gross energy
g	Gravity
mg	Milligram
µg	Microgram
h	Hour
IU	International unit
L	Liter
mL	Milliliter
µL	Microliter
M	Molarity (mol/L)
min	Minute
mm	Millimeter
N	Nitrogen
n	Number
ND	Not determined
NDF	Neutral detergent fiber
NS	Not significant ($P > .05$)
OM	Organic matter
P	Probability
U	Unit
wt	Weight

CHAPTER I

GENERAL INTRODUCTION

A. ß-Glucanase Supplementation to Barley-based Diets for Young Pigs

1. ß-glucans and ß-glucanases

ß-glucans include (1-4)-ß- and (1-3, 1-4)-ß-D-glucans (Annison, 1991). ß-glucans with a regular linear structure are conventionally referred to as cellulose and are present in all plant cell walls primarily to serve as structural components. The mixed linked (1-3, 1-4)-ß-D-glucans, simply referred to as ß-glucans, occur frequently as constituents in the endosperm cell walls of a variety of cereal grains (Anderson et al., 1978). Among the cereal grains, barley and oats are found to contain the highest levels of ß-glucans. ß-glucans in barley differ from cellulose in that about 30% of the linkages between glucose units are of the ß(1-3) and 70% of ß(1-4) type (Fleming and Kawakami, 1977). Both linear and branched linked ß-glucans are resistant to hydrolysis by α-amylase.

Several types of endogenous ß-glucanase have been characterized: (1) endo-(1-3) ß-glucanase; (2) endo-(1-4) ß-glucanase and (3) endo-(1-3, 1-4) ß-glucanase (Bamforth, 1982). The latter is also referred to as endo-barley-ß-glucanase. Endo-(1-3, 1-4)-ß-glucanase activity in barley has been reported by several researchers (e.g., Manners and Marshall, 1969; Ballance et al., 1976; Manners and Wilson, 1976;). Ballance et al. (1976) found that the ß-glucanase activity was primarily located in the embryo and scutellum of barley kernels and it increased markedly during germination. Bathgate and Palmer (1974) suggested that this enzyme is responsible for degrading barley ß-glucans in the early stages of germination. For this reason, endo-ß-glucanase has received considerable attention by the brewery industry.

In addition to the endo-ß-glucanases in barley, some ß-glucanases with different

hydrolyzing capabilities are found in some species of fungi, for example cellulase from *Pennicillium Funiculosum* (Bamforth, 1983), *Trichoderma viride* (Bamforth and Martin, 1979) and *Trichoderma reesi* (Martin and Bamforth, 1981). Some also derived from bacteria, for example ß-glucan endo-hydrolase from *Bacillus subtilis* (Anderson et al., 1978) and Ballance and Manners, 1978) and *Lactobacilli* (Graham et al., 1986).

The aforementioned enzymes are widely employed in numerous procedures for quantification of ß-glucans. These procedures are usually based on the depolymerization of ß-glucans with cellulase or specific endo-(1-3, 1-4)-ß-glucanase followed by glucosidase or acid hydrolysis. The liberated free glucose, on which the quantification of ß-glucans is based, is measured enzymatically by hexokinase and glucose-6-phosphate dehydrogenase system (Martin and Bamforth, 1981), glucose-oxidase (Bamforth et al., 1979) or *p*-hydroxybenzoic acid hydrazide (Henry, 1984).

In many cases, the use of impure or partially purified enzymes may give rise to conflicting results between laboratories. For example, cellulase from *Trichoderma SP.* is very effective in solubilizing ß-glucans in a suspension of barley flour; however, it is also effective in the hydrolysis of amorphous cellulose which leads to an overestimation of ß-glucan content. Consequently, separate samples would have to be analyzed in order to determine glucose that is liberated from ß-glucans and those from amorphous cellulose. In contrast, cellulase from *Aspergillus SP.* does not attack amorphous cellulose, nor does it release free glucose from ß-glucans (McCleary and Glennie-Holmes, 1985). Furthermore, some enzymes can completely hydrolyze ß-glucans to free glucose, for example, ß-glucanase purified from *Trichoderma reesi* (Martin and Bamforth, 1981) and ß-glucanase purified from *Penicillum Funiculosum* (Bamforth, 1983). Others can only hydrolyze ß-glucans to oligosaccharides, for example, carboxypeptidase, which catalyzes the release of soluble ß-glucans from insoluble barley endosperm cell walls (Bamforth et al., 1979). Therefore, additional steps are required to degrade these oligosaccharides to free glucose either by acid hydrolysis (Anderson et al., 1978) or glucose oxidase-peroxidase (GOD/Perid) kit or hexokinase / glucose 6-phosphate dehydrogenase system when more sensitive estimations are required (Bamforth et al., 1979).

The ß-glucanase used in our laboratory was cellulase from *Penicillium Funiculosum* (EC 3.2.1.4, Sigma Chemical Company, St. Louis, MO), which exhibited nearly hundred percent ß-glucanase activity after purification according to the procedure described by Bamforth (1983).

2. Physicochemical properties of ß-glucans

The physicochemical properties of ß-glucans closely related to their physiological and nutritional functions comprise solubility, viscosity and water-holding capacity.

The solubilities of ß-glucans are directly related to their molecular structure. ß-glucans in barley tend to be water soluble, because they are branched linked glucose polymers such as (1-3, 1-4)-ß-D-glucans. The mixed linked ß-glucans differ from linear linked cellulose in that the branched side chains prevent compact folding so that a crystalline array can not be formed. However, this property of the mixed linked ß-glucans is prone to form gels (Oakenfull, 1991).

Viscosity results from physical interactions between the polysaccharide molecules in an aqueous solution and is dependent on molecular weight and whether the molecule is linear or branched (Oakenfull, 1991). Almost all water-soluble polysaccharides produce viscous solutions. Barley ß-glucans have a high ratio of branched to linear structure, 70 vs 30% according to Fleming and Kawakami (1977); these tend to cause a high viscosity of the contents in the digestive tract of some animal species.

Water-holding capacity of polysaccharides is related to the polarity of the free polar groups of sugar residues (Oakenfull, 1991). Polysaccharides are hydrophilic molecules, consequently both water-soluble and insoluble polysaccharides have the ability to hold water. The most obvious demonstration of the ability of soluble polysaccharides to hold water is the phenomenon of gelation, a process where water is trapped in a three-dimensional network of polymer molecules. On the other hand, linear linked cellulose absorbs water in the same manner as a sponge in which water molecules are trapped in a hydrophilic matrix (Oakenfull, 1987).

To summarize, the aforementioned physicochemical properties of ß-glucans exert, either individually or in combination with each other, a detrimental effect on digestion and absorption of nutrients in the digestive tract. The viscosity and gelling properties tend to hinder intestinal motility and slow the diffusion process in the small intestine (Holt et al., 1979), thereby decreasing the mixing of digesta, digestive enzymes and other necessary components required for digestion and absorption (Vahouny and Cassidy, 1985). These properties may also delay or decrease the digestion and absorption of nutrients and energy by increasing the unstirred fluid layer, creating a physical barrier at the absorption surface on the microvilli (Johnson and Gee, 1981).

3. Application of ß-glucanase in swine nutrition

Most studies with ß-glucanase supplementation to barley-based diets for poultry have shown positive responses as was reviewed by Campbell and Bedford (1992). Edney et al. (1989) reported that both growth and feed conversion were improved significantly in chicks (0-3 weeks of age) fed diets containing hulled or hulless barley or oat groats supplemented with ß-glucanase. Similar results were also reported by Hesselman and Åman (1986). Salih et al. (1991) reported that the beneficial effects of ß-glucanase supplementation to hulless barley diets for chicks were largely confined to the early growth period from 0-4 weeks of age. However, studies on ß-glucanase supplementation to barley-based diets for pigs have showed inconsistent results, as summarized in Table I-1.

Some studies have shown improvements in nutrient and energy digestibilities in growing pigs (Graham et al., 1988; Thacker et al., 1988), whereas others have shown no improvement in both young (Bedford et al., 1992; Thacker et al., 1992b) and growing pigs (Graham et al., 1986; Thacker et al., 1989, 1992a). Of these studies only two (Bedford et al., 1992; Thacker et al., 1992b) were carried out with young pigs. Compared with poultry, the lack of a consistent response to ß-glucanase supplementation in pigs may be due to differences in the digestive system. The intestinal contents of pigs are much higher in water content than poultry, which may compromise the detrimental effect of viscosity caused by ß-

glucans. Since the ß-glucan-induced viscosity is logarithmically related to concentration, simple dilution can essentially eliminate the viscosity and its associated constraints on lumenal diffusion (Campbell and Bedford, 1992). In addition, growing pigs are able to degrade ß-glucans to a considerable extent by aid of microorganisms in the small and large intestine. For example, even without supplementation of ß-glucanase, the ileal digestibility of ß-glucans was 80% in studies with pigs of approximately 60 kg BW (Weltzien and Aherne, 1987) and 68% in pigs of 30-50 kg BW (Graham et al., 1986). A higher digestibility value, 95.6%, was reported by Graham et al. (1989) in studies with pigs of 80 kg BW. Further studies showed that microflora, capable of degrading ß-glucans, are virtually completely developed in growing pigs. Graham et al. (1986) observed more than 10^7 lactobacilli per g fresh digesta, including approximately 10^6 with ß-glucan degrading ability, in digesta collected from the duodenum and ileum. From these studies it seems that ß-glucanase supplementation to barley-based diets for growing-finishing pigs has limited nutritional significance.

Considering the physiological differences in the development of the digestive system and microbial population at different stages of growth of the animal, further studies are warranted to investigate the response of ß-glucanase supplementation to diets for very young pigs. ß-glucanase supplementation to hulless barley-based diets that contain relatively high level of ß-glucans, could be benificial in improving nutrient and GE utilization in these pigs.

B. Soybean Trypsin Inhibitors (SBTI) in Swine Nutrition

1. Soybean Trypsin Inhibitors (Kunitz Inhibitor and Bowman-Birk Inhibitor)

Protease inhibitors are widely distributed in legumes, cereals and other plants. Their primary function is associated with the self-defense system of plants (Liener and Kakade, 1980). Protease inhibitors isolated from soybean fall into two major categories, namely Kunitz and Bowman-Birk inhibitors.

The Kunitz inhibitor was first purified and crystallized by Kunitz (1945, 1946, 1947). It has a molecular weight of 20,000-25,000 and consists of 181 amino acid residues and two

disulphide bonds with the reactive site located at residues arginine 63 and isoleucine 64 (Koide et al., 1973). This molecule is thought to have very little α-helical structure and is largely in the form of a random coil. The Kunitz inhibitor exhibits a specificity which is primarily towards trypsin derived from a wide variety of sources, including the cow, pig, salmon, stingray, barracuda and turkey (Kassell, 1970). The Kunitz trypsin inhibitor is unstable at high temperatures and alkaline conditions. It is completely inactivated when autoclaved, using steam, at 100°C for 15 min (Rackis, 1966).

The Bowman-Birk inhibitor was first described by Bowman (1944) and subsequently purified and characterized by Birk and coworkers (Birk, 1961; Birk et al., 1963). It has a molecular weight of 8,000 (Frattali, 1969) and consists of a single polypeptide chain with 71 amino acid residues and seven disulphide bonds (Odani and Ikenaka, 1973). It is rich in cystine and devoid of glycine and tryptophan (Odani and Ikenaka, 1972). This inhibitor exhibits specificity towards both chymotrypsin and trypsin with independent reactive sites. Because of its large number of disulphide bonds, the Bowman-Birk inhibitor is stable both at high temperatures and under acid and alkali conditions (Bowman, 1946; Birk, 1961).

2. Effect of Soybean Trypsin Inhibitors on Exocrine Pancreatic Enzyme Secretions and Protein and Amino Acid Metabolism

Effect on pancreas and enzyme secretions

In studies with a synthetic trypsin inhibitor (*p*-aminobenzamidine), Kakade et al. (1970) observed growth inhibition, enlargement of the pancreas and hypersecretion of pancreatic enzymes in rats, similar to that produced by the natural trypsin inhibitor present in navy beans. In a comprehensive study with rats, Rackis (1965) found that the Kunitz inhibitor accounted for all of the effect on pancreatic hypertrophy and for about 30-60% on growth-inhibition of raw soybean. A decrease in growth rate and metabolizable energy content and hypertrophy of the pancreas were also reported in chicks (Garlich and Nesheim, 1966). One study showed no enterokinase activity in intestinal juice from chicks fed raw

soybean diets (Lepkovsky et al., 1970). These studies demonstrated that several antinutritional factors were present in raw soybean. The most significant detrimental effect of feeding raw soybean is mediated via the pancreas and its enzyme secretions, which in many animal species is shown by hypertrophy of the pancreas and hypersecretion of trypsin and chymotrypsin. However, there are differences between animal species. For example, Gorrill and Thomas (1967) and Gorrill et al. (1967) found poor growth, reduced trypsin and chymotrypsin secretion, but no pancreatic hypertrophy in young calves fed raw soybean. Yen et al. (1977), in studies with pigs fed raw soybean, reported a decrease in the activities of trypsin and chymotrypsin in digesta collected from the small intestine. However, there was no hypertrophy of the pancreas.

With respect to the effect of feeding raw soybean on the pancreas, some studies indicate that enlargement of the pancreas results from hyperplasia of the acinar cells (Kakade et al., 1967, Salman et al., 1968). Other studies show that the enlargement of the pancreas is caused by an increase in cell size (Saxena et al., 1963; Konijn and Guggenheim, 1967). In either case, the morphological changes of the pancreas will lead to an increase in secretion of pancreatic enzymes, in particular the proteolytic enzymes. As a result, coupled with an increased secretion of all pancreatic enzymes into the intestine, particularly trypsin, there will be a higher proteolytic activity and reduced amylase activity in the pancreas. It was postulated that SBTI initiate a regulatory mechanism which increased the synthesis of the proteolytic enzymes in the pancreas. Since SBTI do not stimulate increased synthesis of amylase, a greater depletion of amylase would result from the increased secretion of pancreatic juice. Similar results were reported by Konijn et al. (1970a,b) who observed considerable changes in the enzyme profile in both pancreatic tissue and juice when raw soybean was fed. There was an increase in the synthesis of trypsin and chymotrypsin and a decrease in lipase, elastase and amylase.

Proposed mechanisms regulating exocrine pancreatic enzyme secretion

The mechanisms of regulation of exocrine pancreatic secretions were reviewed by Solomon (1987). A hypothesis for a negative feedback regulation mechanism was proposed by Green and Lyman (1972) and Schneeman et al. (1977). According to their hypothesis, the secretion of enzymes, particularly trypsin and chymotrypsin, are mediated by the intestinal concentrations of these enzymes. Soybean trypsin inhibitors may evoke an increase in pancreatic secretion by removing active trypsin and chymotrypsin from the intestinal contents (via the formation of an enzyme-inhibitor complex), thereby preventing normal feedback regulation.

In contrast to the negative feedback regulation mechanism, a positive feedback regulation mechanism of pancreatic secretions was proposed by Melmed and Boucher (1969). According to this mechanism, pancreatic secretions are regulated by the level of pancreatic trypsin inhibitor released into the intestine. The pancreatic trypsin inhibitor itself provides an important stimulation for the production and depletion of digestive enzymes by the acinar cells. While the negative feedback mechanism initiates stimulation of the pancreas and leads to an increased secretion of pancreatic trypsin inhibitor, the increase in pancreatic trypsin inhibitor, in turn, reinforces and sustains stimulation of the pancreas via a positive feedback mechanism. Consequently, the pancreatic trypsin inhibitor will be expected to reduce the activities of trypsin and chymotrypsin in the intestinal contents in much the same way as SBTI. From this point of view, the pancreatic trypsin inhibitor acts as a participant in the negative feedback regulation mechanism.

Effect on protein and amino acid metabolism

Reports in the literature showed that SBTI influence the metabolism of certain amino acids, particularly cysteine and methionine. Trypsin, which contains 8.7% cystine, accounts for half of the cystine secreted in pancreatic enzymes in rats fed raw soybean (Barnes et al., 1965a). If the ingestion of SBTI stimulates the secretion of trypsin and chymotrypsin, then there will be an increase in the secretion of trypsin when raw soybean, which has a high

content of SBTI, is fed. A higher demand for cysteine by the pancreas creates a deficiency of this amino acid which enhances the conversion of methionine to cysteine. It was observed that the rate of synthesis of cysteine in the pancreas is 7 to 10 times greater than in the liver (Barnes and Kwong, 1965; Barnes et al., 1965b). The enhanced conversion of methionine to cysteine intensifies the need for methionine for body tissue protein synthesis. Moreover, a different hypothesis proposed by Frost and Mann (1966) stated that the deficiency of cysteine created by the ingestion of trypsin inhibitors was due to interference with its incorporation into protein via inhibition of the enzyme cystathionine synthetase.

Studies with rats showed that the ingestion of raw soybean interfered with the catabolism of threonine and valine (Borcher et al., 1965). It was postulated that homoserine, a by-product of conversion from methionine to cysteine, was further catabolized to pyruvate via propionate which is also a common intermediate of threonine and valine catabolism. Since the concentration of propionate will increase when methionine is converted to cysteine, the rate at which threonine and valine are metabolized by the same pathway will be depressed.

3. Studies on Soybean Trypsin Inhibitors with Pigs

In contrast to rats and chicks, studies with pigs (e.g., Yen et al., 1977; Cook et al., 1988) showed that raw soybean caused a decrease in the activities of trypsin and chymotrypsin in intestinal contents, but no hypertrophy of the pancreas. Most studies reported in the literature with pigs focused on performance (e.g., Combs et al., 1967; Yen et al., 1974), nutrient digestibility (Combs et al., 1967; Vandergrift et al., 1983) and enzyme activities in pancreatic tissue and intestinal contents (Yen et al., 1977). One study, with miniature pigs fed raw soybean, reported a reduced absorption of exogenous and increased loss of endogenous protein (Barth et al., 1993). Batterham et al. (1993), in studies with growing pigs that were fed increasing levels of chickpeas and pigeonpeas, reported that the maximum tolerance levels to dietary trypsin and chymotrypsin inhibitors by growing pigs were 4.7 and 4.5 mg/g, respectively. These threshold levels are unlikely to be exceeded in conventional diets containing a large proportion of grain legumes. These high tolerance levels to protease

inhibitors by growing pigs may, in part, explain the lack of pancreatic hypertrophy in pigs. If the function of the pancreas is related to its structure, the inhibition on growth performance, without actually causing pancreatic hypertrophy, by raw soybean may suggest a different mechanism of inhibition in pigs. As was pointed out by Kakade et al. (1976), there appeared to be a direct relationship between the size of the pancreas and the sensitivity of response to raw soybean or SBTI. Species of animals with a pancreas weight exceeding .3% of BW are prone to hypertrophy, whereas those with a ratio below .3% are not prone to hypertrophy when fed raw soybean.

As yet, there is little information on the effect of feeding SBTI on total pancreatic protein and enzyme secretions, which can only be demonstrated with surgically modified pigs. Furthermore, there is a scarcity of information on the effect of SBTI on the ileal digestibilities of amino acids. Therefore, studies were carried out to determine the effect of feeding diets containing relatively high and low levels of SBTI on pancreatic secretions and digestibilities of protein and amino acids in growing pigs.

C. Effect of Dietary Protein Content on Exocrine Pancreatic Secretions in Growing Pigs

There is a scarcity of information on the effect of dietary CP levels on the total secretion of protein and total activities of enzymes in exocrine pancreatic secretions. One study (Hee et al., 1988), with pigs fed a corn starch-based diet containing 15% CP from soybean meal or a corn starch-based protein-free diet, reported no differences ($P > .05$) in the total secretion of protein and lipase. However, the total activities of trypsin, chymotrypsin and amylase were considerably lower ($P < .05$) in pigs fed the protein-free diet. The results by Hee et al. (1988), with respect to the total activities of the aforementioned enzymes, are not surprising because the pigs were in a protein-deficient status. In another study, Corring and Saucier (1972) fed four corn starch-fish meal diets to pigs (0, 10, 30 and 40% CP). Pancreatic protein secretion and enzyme activities were highest in pigs fed the 40% CP diet. However, the results from their study do not allow for the establishment of a relationship

between dietary CP levels and total protein and enzyme activities in pancreatic juice, because the diets were fed in a sequence in which the changes in BW of the animal were not taken into consideration.

Therefore, further studies are required to determine the effect of different dietary CP content on pancreatic secretions in pigs. A study was conducted to determine the effect of dietary CP level (corn starch-based diets containing 12 and 24% CP from soybean meal) on the pancreatic secretion volume, protein content and enzyme activities in growing pigs prepared with the "Pouch" technique (Hee et al., 1985).

D. Objectives of Thesis

Studies included in this thesis are to determine:

1. The effect of ß-glucanase supplementation to cereal-soybean meal diets on the digestibilities of GE, CP and amino acids in young pigs.
2. The effect of SBTI on the exocrine pancreatic secretions: total volume, nitrogen and protein contents, amino acid composition and specific and total activities of digestive enzymes in growing pigs.
3. The effect of dietary SBTI content on nutrient and GE digestibilities in growing pigs.
4. The effect of dietary CP intake on pancreatic secretions: total volume, protein output and specific and total activities of digestive enzymes in growing pigs.

Table I-1. Studies reported in the literature on ß-glucanase supplementation to barley-based diets for pigs

#	Pigs BW(kg)	Diet composition (%) Barley	SBM[A]	ß-glucanase [B]	Digestibilities CP	GE	ß-glucans	Performance ADG	FI	FE	References
1	12.1±0.5	63.2	20.4	0.25	*[1]	ND	ND	*	NS	NS	Bedford et al. (1992)
2	80.0	87.5	10.0	0.50	NS	NS	**	ND	ND	ND	Graham et al. (1989)
3	19-25	53.3	8.6	0.20	*	ND	*	ND	ND	ND	Graham et al. (1988)
4	30-50	61.3	16.5	0.10	NS	NS	NS	ND	ND	ND	Graham et al. (1986)
5	41.2-42.6	76.8	18.3	0.25	NS	NS	NS	NS	NS	NS	Thacker et al. (1992a)
6	8.1±1.3	77.7	15.9	0.25	NS	NS	ND	NS	NS	NS	Thacker et al. (1992b)
7	40±2.0	75-83.5	12.5-21.3	0.25	NS	*	ND	NS	NS	NS	Thacker et al. (1989)
8	40±2.0	88-91.5	5.3-8.7	0.25	*	**	ND	NS	NS	NS	Thacker et al. (1988)

[A]. SBM: soybean meal.

[B] Enzyme activities and sources (in numbered order):

1. ß-glucanase, no further information provided.
2. Activity 20 IU/g, Enzyme Development Corporation, NY.
3. TSE-8, ß-glucanase activity, 16 U/g and xylanase activity, 1,300 U/g. Grindsted Products, A/S, Denmark.
4. Glucanase GV-P, 10,000 U/g. Grindsted Products, A/S, Denmark.
5. ß-glucanase activity, 750 U/g and pentosanase activity, 650 U/g from *Aspergillus niger*, GNC Bioferm Inc., Saskatoon, SK.
6. ß-glucanase activity, 750 U/g from *Aspergillus niger* with dehydrated malt sprouts as carrier. GNC Bioferm Inc., Saskatoon, SK.
7. Refer to 6.
8. Refer to 6.

* : Significant ($P < .05$).

*[1]: Only in digesta collected from the colon.

**: Significant ($P < .01$).

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CHAPTER II

EFFECT OF ß-GLUCANASE SUPPLEMENTATION TO CEREAL-BASED DIETS FOR STARTER PIGS ON THE APPARENT DIGESTIBILITIES OF DRY MATTER, CRUDE PROTEIN AND ENERGY[1]

A. Introduction

The mixed-linked (1-3), (1-4)-ß-D-glucans, which are frequently present in the endosperm cell walls of barley (Åman and Graham, 1987), may interfere with digestion and absorption of nutrients and energy. Although there are physiological reasons for augmenting the digestive capacity of pigs with supplementation of ß-glucanase, the responses to supplementation have been inconsistent. Most studies on ß-glucanase supplementation to barley-based diets have been carried out with growing and finishing pigs. For example, Graham et al. (1986), in studies with 30-50 kg pigs, reported that ß-glucanase supplementation to barley soybean meal diets did not improve ($P > .05$) the duodenal, ileal or fecal digestibilities of GE and CP. Graham et al. (1989), in studies with pigs of 80 kg body weight (BW), found that ß-glucanase supplementation and pelleting did not improve ($P > .05$) ileal and fecal digestibilities of DM, GE and CP, but there was an increase in ileal digestibility of mixed linked ß-glucans ($P < .05$). Further studies (Thacker et al., 1992a), with pigs of initial BW 42 kg and fed barley and rye diets, reported a significant improvement in fecal digestibilities of CP only when ß-glucanase was combined with Salinomycin, whereas no improvement in ADG or FE was found when these two

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[1] A version of this Chapter has been accepted for publication. Li, S., W. C. Sauer, R. Mosenthin, and B. Kerr. 1995. Anim. Feed Sci. Technol. (In press).

supplements were either fed alone or in combination. Only two studies with ß-glucanase supplementation to barley-based diets for starter pigs have been reported in the literature (Bedford et al., 1992; Thacker et al., 1992b).

The objectives of the present studies were to determine the effect of supplementation of ß-glucanase (Cellulase Kyowa, 1,000 fibrinolytic activity units/g) on CP and GE digestibilities in starter pigs fed a hulless barley-soybean meal diet, in addition to wheat-corn- and rye-soybean meal diets.

B. Experimental Procedures

Animals and diets

A total of 48 PIC pigs (Camborough x Canabrid) comprising equal numbers of barrows and gilts, weaned at 3 weeks of age, were obtained from the University of Alberta Swine Research Unit. The average weaning weight of the pigs was 5.9 kg. The pigs were housed individually in metabolic crates (height: 85 cm; length: 70 cm; width: 65 cm) in a barn in which the temperature was maintained between 25 to 28°C. The pigs given *ad libitum* access to an 18% CP starter diet (Sauer et al., 1983). Water was freely available from a low-pressure drinking nipple.

After a period of three days, twelve pigs each (six barrows and six gilts), were assigned to one of four dietary experiments. In each experiment, the pigs were fed hulless barley-, wheat-, corn- or rye-soybean meal diets, respectively. The four dietary treatments were basal diet (control) and basal diet supplemented with .05, .1 and .2% enzyme mixture (Kyowa Hakko Kogyo Co., Ltd, Japan) which consists of enzymes with endo- and exo-ß-glucanase and ß-glucosidase activities up to 1,000 fibrinolytic activity units per gram. One thousand fibrinolytic activity units are equivalent to the amount of cellulase required to degrade completely two pieces of 1 cm^2 filter paper in 1 min at 37° C. Hereafter, this enzyme mixture is referred to as ß-glucanase. Detailed specifications of this enzyme mixture are provided in Appendix I.

Four cereal-soybean meal diets were formulated to contain 20.0% CP (Table II-1).

The barley cultivar used in this study was CDC Buck (an improved six-row hulless barley, released by the Crop Development Center, University of Saskatchewan, Saskatoon, SK). The name of the wheat cultivar, a hard red spring wheat, was unknown. The cereals were ground through a 2-mm mesh screen prior to incorporation into the diets. Solvent-extracted soybean meal (47.3% CP) was used as the protein supplement. Canola oil was included in the diets to meet the NRC (1988) standards for digestible energy. Vitamins, minerals and lysine were also supplemented to meet or exceed the NRC (1988) standards. Chromic oxide (.25%) was used as digestibility marker.

The effect of ß-glucanase supplementation on the digestibilities of DM, CP and GE of the hulless barley soybean meal (B+SBM), wheat soybean meal (W+SBM), corn soybean meal (C+SBM) and rye soybean meal (R+SBM) diets were determined in four independent experiments which were conducted with pigs of similar BW and under the same conditions.

Each of these experiments was carried out according to a balanced two-period changeover design (Gill and Magee, 1976). The allotment of the pigs to the experimental diets is presented in Table II-2. Four dietary treatments were evaluated, i.e. control plus three levels of ß-glucanase supplementation which were represented by A, B, C and D, respectively. The basic plan of this design required six Latin squares of size 2. Each Latin square contained two randomly selected animals as rows and two periods as columns. Therefore, the complete design required 12 animals. The design was balanced because each treatment was compared with any of the other three treatments in two blocks (animals). Experimental periods were orthogonal to dietary treatments This design has advantages in the following situations: (1) complexity and cost demand that the duration of the experiment be as short as possible, yet take advantage of the changeover principle to eliminate variation among animals, (2) animals from a completely randomized first period are re-used in a balanced fashion in a second period to conserve resources and reduce error. However, the disadvantage of this design is its poor control over changes across periods, therefore, interactions between experimental periods and treatments can not be analyzed.

The daily feed allowance was provided at a rate of 5% (wt/wt) of the BW of each pig, which was determined 8 h prior to the start of each experimental period. The average

BW of the pigs were 6.2 kg and 8.1 kg at the start of periods 1 and 2, respectively. The average BW of the pigs at the conclusion of the experiment was 11.2 kg. The pigs were fed three times daily, equal amounts, at 0800, 1600 and 2400. All pigs consumed their meal allowances within 2 h after feeding. Each experimental period lasted 10 days. Following a 7-d adaptation period, feces were collected from 0800 on d 8 until 0800 on d 11. Feces were frozen at -20° C immediately after collection.

The animals used in these experiments were cared for in accordance with the guidelines established by CCAC (1980).

Chemical and statistical analyses

After the conclusion of the experiment, feces were pooled within pig and period for the same dietary treatment. Samples were oven-dried at 65°C for three days, ground in a Wiley mill (Arther H. Thomas Co., Philadelphia, PA) through a .8-mm mesh screen and stored at 4° C.

Analyses for DM and CP were carried out according to the procedures of the AOAC (1984). Analysis for GE was carried out with a Parr Adiabatic Bomb Calorimeter. Analyses for NDF and ADF were carried out according to principles outlined by Goering and van Soest (1970). Chromic oxide was determined according to Fenton and Fenton (1979). The contents of ß-glucans in the cereal grains, soybean meal and the diets were determined according to a procedure based on Bamforth (1983) and Henry (1984) with modifications (Appendix II).

Samples of cereal grains, soybean meal and diets were ground through .3-mm mesh screen; .1 g of sample was weighed out and suspended in 80% ethanol and gently boiled for 5 min in a water bath and washed several times with ethanol. The purpose of extraction with ethanol combined with gentle heating was to inactivate endogenous α-amylase and amyloglucosidase and also to remove free glucose. After the samples were cleaned up, the ß-glucans remaining in the pellet were hydrolyzed with ß-glucanase from *Penicillium Funiculosum* (Sigma Chemical Company, St. Louis, MO; Catalog C-0901), which was purified according to procedures described by Henry (1984). After incubation of the samples

with ß-glucanase, in a water bath at 40° C for 2 h, the reducing sugars liberated from ß-glucans were measured enzymatically with a hexokinase and glucose-6-phophate dehydrogenase kit (Sigma, Glucose Diagnostic, Catalog 115-A). The optical density was measured colorimetrically at 520 nm with a Milton Roy Spectronic 3000 Array Spectrophotometer equipped with an auto-sampling pump (Fisher Scientific, Edmonton, AB, Canada).

A ß-glucan standard curve was prepared under the same conditions as described for the samples with barley ß-glucan as standard (Sigma, Catalog G-6513). The preparation of the standard solution is shown in Table A2-1 and the standard curve in Figure A2-1. To verify the purity of the ß-glucanase preparation, a series of amylose standards from potatoes (Sigma, type III, lot 42H3861) were also prepared using the same concentration and under the same incubation conditions as the ß-glucan standards. Results showed that there was no glucose produced in the amylose standards, indicating that enzyme purification had minimized the α-amylase and amyloglucosidase activities.

ß-glucan content in the sample was calculated using the following formula:

ß-glucans (mg/g sample) = [(A_{520} - .083)/.098]/sample wt (g)

Where A_{520} = Net absorbance of sample at 520 nm.

All analyses were carried out in duplicate. The partial chemical analysis of the experimental diets is presented in Table II-3.

Data were subjected to statistical analysis using the General Linear Model of SAS (1988). The following linear model was used for data analysis:

$$Y = \mu + T_i + P_j + B_k + \epsilon_{ijk}$$

[i = 1, 2...T; j = 1, 2; k = 1, 2,...T(T-1)].

Where T_i = fixed effect of treatment.

P_j = fixed effect of experimental period.

B_k = random effect of an animal.

ϵ_{ijk} = systematic error with N(0,1).

Means of treatments and periods were compared using the Student Newman- Keuls' multiple range test procedure and the statistical significance level was claimed at $P < .05$;

the linear, quadratic and cubic effects of the treatments were analyzed according to the orthogonal polynomial regression procedure (Steel and Torrie, 1980). Since the quadratic and cubic effects were not significant ($P > .05$), they were excluded from the model and only the linear regression model $Y = a + bx$ was applied.

C. Results and Discussion

All pigs remained healthy and consumed their meal allowances throughout the experiment.

The digestibilities of DM, CP and GE in the B+SBM diet increased linearly ($P < .05$) with increasing levels of ß-glucanase supplementation (Table II-4). The digestibility of GE increased ($P < .05$) from 85.2 to 89.5% and the digestibility of CP ($P < .05$) from 81.6 to 88.5% when .2% ß-glucanase was supplemented. There was no effect ($P > .05$) of ß-glucanase supplementation on the digestibilities of DM, CP and GE in the W+SBM, C+SBM and R+SBM diets.

The aforementioned results were anticipated. As shown in Table II-3, among the diets, the content of ß-glucans was highest in the B+SBM diet (3.6%). The hulless barley used in this study (*c.v.* CDC Buck) contained a relatively high level of ß-glucans (5.8%), whereas the other diets contained much lower levels of ß-glucans. ß-glucans, which are frequently present in the endosperm cell walls of barley, may interfere with digestion (Anderson et al., 1978). With supplementation of ß-glucanase, there is an increase in the breakdown of endosperm cell wall components, resulting in more complete digestion of starch and protein in the small intestine (Hesselman and Åman, 1986). The mechanisms by which ß-glucans interfere with digestion and absorption are closely related to their physicochemical properties. ß-glucans differ from cellulose in that approximately 30% of the linkages between glucose units are in the form of ß (1-3) and 70% in the form of ß (1-4) (Fleming and Kawakami, 1977). This branched structure prevents compact folding of the molecules and increases the water-holding capacity which in turn results in its characteristic viscosity and gelling properties. The viscosity and gelling properties tend to hinder intestinal

motility (Holt et al., 1979) thereby decreasing the mixing of digesta, digestive enzymes and other necessary components required for digestion and absorption (Vahouny and Cassidy, 1985). These properties may also delay or decrease the digestion and absorption of nutrients by increasing the unstirred fluid layer, creating a physical barrier at the absorption surface on the microvilli (Johnson and Gee, 1981).

With the exception of studies by Thacker et al. (1992b), in which no improvements ($P > .05$) were obtained in the digestibilities of GE and CP, no other total tract digestibility studies with ß-glucanase supplementation to barley-based diets in young pigs have been reported in the literature. Bedford et al. (1992), using the slaughter method in studies with young pigs (12.1 kg), reported an improvement in nitrogen digestibility in digesta collected from the colon ($P < .05$), however, no significant improvement was found in digesta collected from the last three quarters of the small intestine ($P > .05$). Total tract digestibilities were not determined in the aforementioned studies. In studies with older pigs fed barley-based diets (30-50 kg), Graham et al. (1986) observed no effect ($P > .05$) of ß-glucanase supplementation on CP and GE digestibilities. Similarly, Graham et al. (1989) found no effect ($P > .05$) of ß-glucanase supplementation on ileal as well as fecal digestibilities of CP and GE in studies with 80 kg pigs. Furthermore, Thacker et al. (1992a), in studies with pigs of 41-43 kg, found no effect ($P > .05$) on CP and GE digestibilities. On the other hand, in one study with growing pigs (40 kg), improvements ($P < .05$) were reported in GE and CP digestibilities (Thacker et al. 1988) upon ß-glucanase supplementation.

It is rather difficult to consolidate the digestibility results from the different studies. However, in all but one study with older pigs, there was no effect of ß-glucanase supplementation on CP and GE digestibilities which may explain the lack of response in performance in most of the studies with older pigs (Thacker et al., 1989; Thacker et al., 1992a). In this context, Graham et al. (1986), in studies with pigs weighing 30-50 kg, observed more than 10^7 *lactobacilli* per gram fresh digesta, including about 10^6 with ß-glucan degrading ability, in digesta collected from the duodenum and ileum. The ileal ß-glucan digestibilities in this study ranged from 95.7 to 97.1%, similar to that observed by

Graham et al. (1989) in studies with 80 kg pigs. In addition, Weltzien and Aherne (1987), in studies with pigs of 57.6 kg, reported an ileal β-glucan digestibility of 80%. Furthermore, studies by Graham et al. (1986) and Graham et al. (1989) also showed that ß-glucans were completely degraded by the microflora in the large intestine. Based on the previous discussion, it is unlikely that ß-glucanase supplementation to barley-based diets for older pigs will improve nutrient and GE digestibilities and performance.

At present, there is a scarcity of information on the effect of ß-glucanase supplementation on nutrient and GE digestibilities to barley-based diets for young pigs. The results from this study and to a lesser extent from Bedford et al. (1992) showed a positive effect while Thacker et al. (1992b) showed no effect. With respect to performance, Bedford et al. (1992) also showed an improvement ($P < .05$) in ADG upon ß-glucanase supplementation.

Some caution should be exercised in the interpretation of results obtained in studies with enzyme supplementation. Various factors should be taken into account. These include the source and activity of the enzyme preparations and the barley cultivars. Commercial enzyme products from different companies may vary in purity and, therefore, enzyme activity, even if the products have the same name. Furthermore, as was shown by Campbell et al. (1989), the ß-glucan content and the extract viscosity of barley were influenced by the genotype and environment. In their studies, 16 barley cultivars representing high or low viscosity traits were grown at five different locations across western Canada. The results showed that there was an interaction between viscosity genotype and location; low viscosity barleys did not differ in viscosities among locations whereas high viscosity barleys did ($P < .01$). Endogenous ß-glucanase levels were consistently lower for high than low viscosity barleys. Protein contents were not closely related to viscosity. The effectiveness of enzyme supplementation is directly related to differences in endogenous ß-glucanase levels and extract viscosity of the barley.

The digestibilities of DM, CP and GE were higher in period 2 than 1 (Table II-5). The differences, although of a small magnitude, were significant ($P < .05$) for the B+SBM, W+SBM and C+SBM diets. These results are in agreement with studies in young pigs

reported by Lloyd et al. (1957) and Li and Sauer (1994). The increase in digestibility with age may result from further development of the digestive tract and increased microbial activity. Kidder and Manners (1978) reported an increase in digestive enzyme secretion, with the exception of lactase, up to 7-8 weeks of age.

In conclusion, ß-glucanase supplementation to the B+SBM diet improved ($P < .05$) the digestibilities of DM, GE and CP in young pigs. There was no effect ($P > .05$) of ß-glucanase supplementation to the W+SBM, C+SBM or R+SBM diets. Further studies on ß-glucanase supplementation to barley-based diets for young pigs are warranted. These include the determination of ileal amino acid digestibilities and ß-glucan degrading activity of the microflora in the digestive tract of young pigs.

Table II-1. Formulation (%)[a] of hulless barley-, wheat-, corn- and rye-soybean meal basal diets

	Basal Diets[b]			
Ingredients	B+SBM	W+SBM	C+SBM	R+SBM
Barley	59.83			
Wheat		69.90		
Corn			65.67	
Rye				59.29
Soybean meal	29.30	23.38	29.94	32.92
Canola oil	6.80	2.56	.34	3.90
Dicalcium monophosphate	1.40	1.34	1.38	1.46
Vitamin-mineral premix[c]	1.00	1.00	1.00	1.00
Calcium carbonate	.83	.92	.87	.78
Trace-mineralized salt[d]	.30	.30	.30	.30
Chromic oxide[e]	.25	.25	.25	.25
L-Lysine HCL	.19	.25	.15	.00
Antibiotics[f]	.10	.10	.10	.10

[a] As-fed basis.

[b] The four basal diets were formulated based on hulless barley plus soybean meal (B+SBM), wheat plus soybean meal (W+SBM), corn plus soybean meal (C+SBM) and rye plus soybean meal (R+SBM), respectively.

[c] Provided (per kg diet): Vitamin A, 10,000 IU; vitamin D_3, 1,000 IU; vitamin E, 80 IU; vitamin K_3, 2.0 mg; vitamin B_{12}, .03 mg; riboflavin, 12 mg; niacin, 40 mg; pantothenic acid, 25 mg; choline, 1,000 mg; biotin, .25 mg; folic acid, 1.6 mg; thiamine, 3.0 mg; Ethoxyquin, 5.0 mg; pyridoxine, 2.25 mg. Fe, 150 mg; Zn, 150 mg; Cu, 125 mg; I, .21 mg; Se, .3 mg. Supplied by Hoffmann-LaRoche Ltd., 2455 Meadowprine Bvd., Mississauga, ON.

[d] Provided (%):$Ca(IO_3)_2$, .007;CaO, .004; CuO, .033; $FeCO_3$, .16; MnO, .12; NaCl, 96.5; ZnO, .4; Supplied by Windsor Salt, Toronto, ON.

[e] Fisher Scientific. Fair Lawn, NJ, 07410.

[f] ASP 250 mixture, provided (mg/kg mixture): Aureomycin 100, Sulfamethazine 100 and Penicillin 50. Supplied by The Upjohn Company, Animal Health Division, Orangeville, ON.

Table II-2. Balanced two-period change-over design[a]

Animal (block)	Period 1	Period 2
1	A	B
2	B	A
3	A	C
4	C	A
5	A	D
6	D	A
7	B	C
8	C	B
9	B	D
10	D	B
11	C	D
12	D	C

[a] A, B, C and D represent the four levels of ß-glucanase supplementation at 0, .05, .1, and .2%, respectively.

Table II-3. Partial chemical analysis of the hulless barley-, wheat-, corn- and rye-soybean meal basal diets (%)[a]

Items	Basal Diets			
	B+SBM	W+SBM	C+SBM	R+SBM
Dry matter	89.8	88.2	88.2	88.4
Crude protein	21.7	21.8	21.1	22.0
Gross energy (Mcal/kg)	4.3	4.1	3.9	4.1
Neutral-detergent fiber	9.4	8.9	9.2	8.4
Acid-detergent fiber	2.6	2.8	2.2	2.4
Ether extract	8.4	3.9	3.0	5.2
ß-glucans[b]	3.6	.9	.3	.8

[a] Dry matter basis.

[b] ß-glucan contents of barley, wheat, corn, rye and soybean meal were 5.8, 1.1, .4, 1.2 and .3%, respectively.

Table II-4. Effect of ß-glucanase supplementation on the apparent digestibilities (%) of dry matter, crude protein and gross energy in starter pigs

Diets		Level of ß-glucanase supplementation (%)				
		.00	.05	.10	.20	SE[a]
B+SBM	Dry matter[b]	84.7[d]	87.1[cd]	86.0[cd]	88.3[c]	.63
	Crude protein[b]	81.6[d]	86.0[cd]	83.4[d]	88.5[c]	1.21
	Gross energy[b]	85.2[d]	87.8[cd]	86.4[cd]	89.5[c]	.87
W+SBM	Dry matter	86.7	87.7	87.8	87.7	.41
	Crude protein	85.1	87.8	88.2	89.1	.91
	Gross energy	86.8	88.1	88.4	88.4	.37
C+SBM	Dry matter	85.6	84.1	83.7	85.2	.67
	Crude protein	84.4	82.5	81.3	82.7	.98
	Gross energy	85.8	84.4	83.8	85.7	.70
R+SBM	Dry matter	86.6	87.2	87.2	86.4	.52
	Crude protein	87.0	88.4	89.3	87.1	1.02
	Gross energy	87.2	88.0	88.1	87.1	.61

[a] Standard error of the mean (n = 6).

[b] Linear effect ($P < .05$).

[c,d] Values in the same row with different superscript letters differ ($P < .05$).

Table II-5. Effect of experimental period on the apparent digestibilities (%) of dry matter, crude protein and gross energy in starter pigs

Diets		Period 1	Period 2	SE[a]
B+SBM	Dry matter	85.4[c]	87.6[b]	.37
	Crude protein	83.2[c]	86.6[b]	.78
	Gross energy	86.0[c]	88.4[b]	.56
W+SBM	Dry matter	86.6[c]	88.4[b]	.29
	Crude protein	86.2[c]	88.9[b]	.64
	Gross energy	87.2[c]	88.6[b]	.26
C+SBM	Dry matter	82.2[c]	87.2[b]	.47
	Crude protein	80.3[c]	85.1[b]	.69
	Gross energy	82.4[c]	87.5[b]	.49
R+SBM	Dry matter	86.4	87.3	.37
	Crude protein	87.2	88.7	.72
	Gross energy	87.2	87.9	.43

[a] Standard error of the mean (n = 12).

[b,c] Values in the same row with different superscript letters differ ($P < .05$).

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CHAPTER III

EFFECT OF ß-GLUCANASE SUPPLEMENTATION TO HULLESS BARLEY- OR WHEAT-SOYBEAN MEAL DIETS ON THE DIGESTIBILITIES OF ENERGY, CRUDE PROTEIN, ß-GLUCANS AND AMINO ACIDS IN YOUNG PIGS[1]

A. Introduction

Barley is usually not included in diets for young pigs, however, the development of hulless cultivars of barley with a high digestible energy (DE) content may result in increased usage by young pigs. It has been reported that hulless barley contains higher levels of ß-glucans compared with hulled barley (Campbell et al., 1986). ß-glucans, which are present in the endosperm and aleurone cell walls of barley, may interfere with digestion and absorption (Åman and Graham, 1987). There is scarcity of information on the effect of ß-glucanase supplementation on the digestibilities of nutrients and GE in barley-based diets for young pigs, which as suggested by Graham et al. (1988), may benefit most from ß-glucanase supplementation.

The DE content, measured with the fecal analysis method, provides no information on the relative disappearance of energy in the small and large intestine, which affects the efficiency of energy utilization (Just et al., 1983). Furthermore, the digestible ileal rather than digestible fecal amino acid supply, should be considered in feed evaluation (e.g., Sauer and Ozimek, 1986).

The objectives of this study were to determine the effect of ß-glucanase supplementation on both the ileal and fecal GE and amino acid digestibilities in young pigs

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[1] A version of this Chapter has been accepted for publication. S. Li, W. C. Sauer, S. X. Huang and, V. M. Gabert. J. Anim. Sci.

fed a hulless barley-soybean meal diet, in addition to a wheat-soybean meal diet. Because a trend towards an improvement in fecal GE and CP digestibility upon ß-glucanase supplementation to a wheat-soybean meal diet was observed in our previous study (Li et al., 1995), wheat-soybean diet was also included in this study to further determine the effect of ß-glucanase supplementation on ileal digestibilities of wheat-based diet.

B. Experimental Procedures

Animals and diets

Twelve PIC barrows (Canabrid x Camborough), weaned at three weeks of age with an average body weight (BW) of 7.1 ± 0.9 kg, were obtained from the University of Alberta Swine Research Unit. The barrows were housed individually in metabolic crates (height: 85 cm; length: 70 cm; width: 65 cm) in a barn in which the temperature was maintained between 25 and 28° C. The pigs were given *ad libitum* access to a starter diet containing 18% CP (Sauer et al., 1983). Water was freely available from a low-pressure drinking nipple.

The pigs were fitted with a simple T-cannula at the distal ileum on d 6 and 7 after weaning. A detailed description of cannula preparation, surgery, pre- and post-operative care was previously provided by Li et al. (1993).

Two basal diets, consisting of hulless barley-soybean meal (B+SBM) or wheat-soybean meal (W+SBM), were formulated (Table III-1). The barley cultivar used in this study was CDC Buck hulless barley which is the same barley used in the previous study (Chapter II). The name of the wheat cultivar, a hard red spring wheat, was unknown. The cereals were ground through a 2-mm mesh screen prior to incorporation into the diets. Solvent-extracted soybean meal (47.3% CP) was used as protein supplement. Canola oil was included in the diets to increase the DE content to the level recommended by NRC (1988). Vitamins, minerals and lysine were also supplemented according to NRC (1988) standards. Chromic oxide (.3%) was used as a digestibility marker. To each of the two basal diets, .2% "Cellulase" (Kyowa Hakko Kogyo, Co., Ltd., Japan) was supplemented. The "Cellulase "

consists of a mixture of enzymes with endo- and exo-ß-glucanase and ß-glucosidase activities up to 1,000 fibrinolytic activity units per gram. One thousand fibrinolytic activity units are equivalent to the amount of cellulase required to completely degrade two pieces of filter paper of 1 cm^2 each in 1 min at 37°C. Hereafter, this enzyme mixture is referred to as ß-glucanase. The specifications of this enzyme preparation is described in Appendix I.

Six pigs were randomly allotted to the B+SBM basal and enzyme supplemented diets (Exp. 1); another six to the W+SBM basal and enzyme supplemented diets (Exp. 2). Both experiments were carried out according to a two-period crossover design (Petersen, 1985). The daily feed allowance was provided at a rate of 5% (wt/wt) of the individual BW which was determined at the start of each experimental period. The average BW of the pigs were 7.3 ± .8 and 9.2 ± 1.2 kg at the beginning of periods 1 and 2, respectively. The average BW at the conclusion of the experiment was 11.4 ± 1.8 kg. The pigs were fed three times daily at 0800, 1600 and 2400, equal amounts each meal in the form of mash. All pigs consumed their meal allowance within 2 h after feeding.

Each experimental period lasted nine days. After a 5-d adaptation period, feces were collected for 48 h: from 0800 on d 6 until 0800 on d 8. Ileal digesta were collected for 24 h: from 0800 to 1600 on d 8, from 2400 on d 8 to 0800 on d 9, and from 1600 to 2400 on d 9. The procedures for collection of feces and digesta were previously described by Li et al. (1993). Soft plastic tubing (20 x 4 cm), sealed at one end and filled with 10 mL of 4% formic acid to stop bacterial activity, was attached to the barrel of the cannula and tightened with Velcro tape. Before the collection tube was attached, the inside of the barrel of the cannula was flushed with saline to facilitate the flow of digesta. Feces samples were collected fresh. Both feces and digesta samples were frozen at -20°C immediately after collection. The samples were pooled leaving one sample of feces and digesta for each pig for each experimental period. The pigs used in these experiments were cared for in accordance with the guidelines established by CCAC (1980).

Chemical and Statistical Analyses

Samples of the diets were taken each time the meal allowances were weighed out and finally pooled for each dietary treatment. Feces and ileal digesta were freeze-dried and ground in a Wiley mill through a .8-mm mesh screen before analysis. Samples of ingredients and diets were ground similarly. Analyses for DM and OM were carried out according to AOAC (1984). Gross energy was determined using a Parr 1241 Adiabatic Bomb Calorimeter (Parr Instruments, Moline, IL). Nitrogen was determined with a Leco FP-428 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI). Chromic oxide was measured according to Fenton and Fenton (1979). Analyses for NDF and ADF were carried out according to principles outlined by Goering and van Soest (1970). For amino acid analysis, approximately .1 g of sample was weighed into a screw-capped test tube (13 x 100 mm) and mixed with 3 mL 6 N HCL. The tubes were flushed with nitrogen and then hydrolyzed in an oven at 110° C for 24 h. An internal standard, DL-amino-n-butyric acid, was added to the hydrolysate, mixed with a Vortex Genie™ and centrifuged at 1,110 x g for 15 min. The supernatant of the sample was analyzed according to principles outlined by Jones and Gilligan (1983) with modifications by Sedgwich et al. (1991) using a Varian 5000 high performance liquid chromatography system with a Varian Flourichrom detector (Varian Canada, Inc., Mississauga, ON). The sytem was equipped with a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Supelco, Oakville, ON) and a guard column (4.6 x 50 mm).The samples were injected using a Valco auto-injector valve equipped with a 20 uL loop. After derivatization, chromatographic peaks were recorded with a Fisher Recordall recorder and integrated using Ezchrom computer software. The mobile phase consisted of two solvents with a total flow rate of 1.1 mL/min. Solvent A contained .1 M sodium acetate (pH 7.2) : methanol : tetrahydrofuran in a ratio of 905:90:5; solvent B was methanol. Total analysis time was 35 min. Methionine, cysteine, proline and tryptophan were not determined. Samples of feces and digesta were analyzed in duplicate; diets and ingredients were analyzed in triplicate.

The contents of ß-glucans in the cereal grains, soybean meal and the diets were determined according to procedures based on Bamforth (1983) and Henry (1984) with

modifications (Appendix 2). The samples of barley, wheat, diets and soybean meal were ground through .3-mm mesh screen; approximately .1 g of sample was weighed and suspended in 5 mL 80% ethanol and gently boiled for 5 min in a water bath and washed several times with this ethanol. The purpose of extraction with ethanol combined with gentle heat was to inactivate the endogenous α-amylase and amyloglucosidase and also to remove free glucose. After the samples were cleaned up, the ß-glucans remaining in the pellet were hydrolyzed with ß-glucanase from *Penicillium Funiculosum* (Sigma Chemical Company, St. Louis, MO; Catalog C-0901), which was purified according to procedures described by Henry (1984). After incubation of samples with ß-glucanase in a water bath at 40° C for 2 h, the reducing sugars liberated from ß-glucans in the samples were measured enzymatically with a hexokinase and glucose-6-phosphate dehydrogenase kit (Sigma, Glucose Diagnostic, Catalog 115-A). The optical density was measured colorimetrically at 520 nm with a Milton Roy Spectronic 3000 Array Spectrophotometer equipped with an auto-sampling pump (Fisher Scientific, Edmonton, AB, Canada).

A ß-glucan standard curve was prepared under the same condition as the samples using barley ß-glucan as standard (Sigma, Catalog G-6513). The preparation of the standard solution is shown in Table A2-1 and the standard curve in Figure A2-1. The purity of the ß-glucanase preparation was verified using amylose standards (Sigma: type III; from potatoes) which were prepared using the same concentrations and under the same incubation conditions as the ß-glucan standards. Results showed that glucose liberated from the amylose standards was negligible, indicating that the enzyme purification had reduced the α-amylase and amyloglucosidase activities to a minimum. The detailed procedure for ß-glucan determination is described in Appendix 2.

ß-glucan content was calculated using the following formula:

ß-glucans (mg/g sample) = [(A_{520}-.083)/.098]/sample wt (g).

Where A_{520} = Net absorbance at 520 nm (A_{520} sample - A_{520} blank).

All analyses were carried out in duplicate. The chemical analysis and amino acid compositions of the experimental diets are presented in Table III-2.

Analysis of variance was carried out according to Petersen (1985) with dietary

treatments, experimental periods and the interaction of treatments and periods as sources of variation using the General Linear Model procedure of SAS (1990). The least square means of treatments and periods were compared using the *t*-test (Steel and Torrie, 1980).

C. Results and Discussion

The pigs remained healthy and consumed their daily allowances throughout the experiment. Postmortem examinations, at the conclusion of the experiment, revealed no intestinal adhesions or other abnormalities.

The supplementation of ß-glucanase to the B+SBM diet improved ($P < .05$ or $P < .01$) the ileal digestibilities of DM, OM, CP, GE and ß-glucans, as well as the majority of the amino acids (Table III-3). Of the indispensable amino acids, with the exception of lysine, the increase ($P < .05$ or $P < 01$) in apparent digestibilities ranged from 4.9 (arginine) to 9.8 (threonine) percentage units. The increase in ileal digestibilities of the parameters measured was also reflected by an increase in the digestibility of ß-glucans in the small intestine from 80.1 to 92.1%. Graham et al. (1986), in studies with older pigs (30 to 50 kg), reported no effect ($P > .05$) of ß-glucanase supplementation to a barley-based diet on the ileal digestibilities of GE and CP as well as non-starch polysaccharides. Similarly, Graham et al. (1989), in studies with 80 kg pigs, found no effect on the ileal digestibilities of GE, CP and dietary fiber components. The difference in results from this and the aforementioned studies may be ascribed, in part, to differences in age of the pigs. Graham et al. (1988), suggested that the fiber-degrading capacity increases with age in the small intestine of the pig. ß-glucanase supplementation may, therefore, be more beneficial to diets for early-weaned rather than for growing or finishing pigs.

Differences in response to ß-glucanase supplementation between different studies may also depend on the ß-glucan content of the diet (Table III-2). The B+SBM diet contained 3.8% ß-glucans which resulted from its high content in the hulless barley (5.8%) that was used in these studies. Åman and Graham (1987) reported an average content of 4.5% ß-glucans in barley, with a range from 3.0 to 6.9% (DM basis). ß-glucans, present in

barley endosperm and aleurone cell walls, may interfere with digestion (Anderson et al., 1978). With the supplementation of ß-glucanase, there is an increase in the breakdown of cell wall components, resulting in more complete digestion of starch and protein in the small intestine (Hesselman and Åman, 1986). The mechanisms by which ß-glucans interfere with digestion and absorption are closely related to their physicochemical properties. ß-glucans differ from cellulose in that approximately 30% of the linkages between glucose units are in the form of ß(1-3) and 70% in the form of ß(1-4) (Fleming and Kawakami, 1977). This branched structure prevents compact folding of the molecules and increases the water-holding capacity which results in its characteristic viscosity and gelling properties. The viscosity and gelling properties tend to hinder intestinal motility (Holt et al., 1979) thereby decreasing the mixing of digesta, digestive enzymes, and other necessary components required for digestion and absorption (Vahouny and Cassidy, 1985). These properties may also delay or decrease digestion and absorption of nutrients by increasing the unstirred fluid layer, creating a physical barrier at the absorption surface on the microvilli (Johnson and Gee, 1981).

This study shows that, even without supplementation of ß-glucanase, there is considerable degradation of ß-glucans in the small intestine of the young pig (Table III-3). Degradation of ß-glucans in the small intestine was previously documented in studies with older pigs fed barley-based diets. For example, Graham et al. (1986) and Graham et al. (1989) reported ileal digestibilities of 68.0 (without ß-glucanase supplementation) and 72.3% (with ß-glucanase supplementation) in studies with pigs from 19 to 25 kg and 95.6 (without) and 97.2% (with) in studies with pigs of 80 kg, respectively.

With the exception of ß-glucans, the supplementation of ß-glucanase to the B+SBM diet also increased ($P < .05$ or $P < .01$) the fecal digestibilities of the parameters that were measured (Table III-3). The CP and GE digestibilities increased by 5.4 and 2.6 percentage units, respectively. The increases in the apparent digestibilities of the indispensable amino acids ranged from 2.5 (leucine) to 4.9 (threonine) percentage units. Previous studies (Chapter II), with 5 - 6 week old pigs, also showed improvements ($P < .05$) in fecal CP (81.6 to 88.5%) and GE (85.2 to 89.5%) digestibilities when .2% ß-glucanase was supplemented

to a 20% CP barley-soybean meal diet. Conversely, Thacker et al. (1992), in studies with 7 to 8 week old pigs, reported no effect of ß-glucanase supplementation on the fecal digestibilities of CP and GE. The effect of ß-glucanase supplementation to barley-soybean meal diets for pigs over 20 kg BW was previously reviewed in Chapter II. In most of the studies, there was no effect of ß-glucanase supplementation on the fecal CP and GE digestibilities.

The ileal as well as fecal digestibilities of all parameters measured were higher for the ß-glucanase supplemented W+SBM diet (Table III-4). However, the differences were only significant ($P < .05$) for the ileal digestibility of ß-glucans and fecal digestibility of GE. Previous studies (Chapter II) also showed higher fecal digestibilities of GE and CP, albeit not significant ($P > .05$).

Results of the present study showed that there was a lower ($P < .05$) net disappearance of DM, OM, GE and ß-glucans in the large intestine of pigs fed the ß-glucanase supplemented B+SBM diet. The net disappearance of CP and amino acids followed the same pattern but the differences were not significant (Table III-5). A similar pattern was observed when ß-glucanase was supplemented to the W+SBM diet. Of the indispensable amino acids, the net disappearance in the large intestine was highest for leucine and threonine; of the dispensable amino acids, for aspartic acid, glutamic acid and glycine which is in agreement with previous studies (Li et al., 1993; Li and Sauer, 1994). As can be inferred from the aforementioned results, ß-glucanase supplementation resulted in an increase in digestion and absorption of nutrients and energy in the small intestine and a decrease in microbial fermentation of these in the large intestine. This shift in the net disappearance of energy and nutrients from the large to the small intestine, in this case, resulted from ß-glucanase supplementation, will also result in an improvement in the efficiency of energy utilization as was shown by Just et al. (1983) in studies with pigs fed diets differing in fiber content. In particular, this modification in energy utilization may be beneficial to young pigs fed diets high in non-starch polysaccharides, in which energy intake may be a limiting factor.

The ileal as well as fecal digestibilities of all the parameters measured, in both the

B+SBM and W+SBM diets, were higher in period 2 than in period 1 (results not shown), which is in agreement with other studies with young pigs (Li and Sauer, 1994). However, the differences were not significant ($P > .05$) in the present study.

In conclusion, the supplementation of ß-glucanase to the B+SBM diet improved ($P < .05$ or $P < .01$) the ileal digestibilities of DM, OM, GE, CP, ß-glucans and the majority of amino acids and the fecal digestibilities of GE, CP and all amino acids that were measured. With the exception of the fecal digestibility of GE which improved slightly, there was no effect ($P > .05$) of ß-glucanase supplementation to the W+SBM diet on the ileal as well as fecal digestibilities of GE, CP and amino acids.

D. Implications

The supplementation of ß-glucanase to a barley-soybean meal diet, high in ß-glucan content, for young pigs has a positive effect on the digestibilities of GE, CP and amino acids. Supplementation of ß-glucanase also shifts the disappearance of GE from the large to the small intestine which may result in an improvement in the efficiency of GE utilization.

Table III-1. Formulation (%)[a] of hulless barley- and wheat-soybean meal basal diets for young pigs

Ingredients:	Diets[b]	
	B+SBM	W+SBM
Barley	62.00	--
Wheat	--	68.50
Soybean meal	27.00	24.00
Canola oil	6.60	3.10
Calcium carbonate	1.35	1.38
Biophos[c]	1.27	1.16
Vitamin-Mineral premix[d]	1.00	1.00
Trace-mineralized salt	.30	.30
Chromic oxide	.30	.30
Antibiotics[e]	.10	.10
Lysine-HCL	.08	.16

[a] As-fed basis.

[b] B+SBM: hulless barley+soybean meal diet; W+SBM: wheat+soybean meal diet. ß-glucanase, .2%, was supplemented to each experimental diet.

[c]. Provided (%): available phosphorous, 15-18 and calcium, 24. Supplied by Continental Lime Ltd., Exshaw, AB.

[d] Provided the following (per kg diet): vitamin A, 10,000 IU; vitamin D_3, 1,000 IU; vitamin E, 80 IU; vitamin K_3, 2.0 mg; vitamin B_{12}, .03 mg; riboflavin, 12 mg; niacin, 40 mg; pantothenic acid, 25 mg; choline, 1,000 mg; biotin, .25 mg; folic acid, 1.6 mg; thiamine, 3.0 mg; Ethoxyquin, 5.0 mg; pyridoxine, 2.25 mg. Fe, 150 mg; Zn, 150 mg; Cu, 125 mg; I, .21 mg; Se, .3 mg. Supplied by Hoffmann-LaRoche Ltd., 2455 Meadowprine Blvd., Mississauga, ON.

[e] Veterinary LS-20 premix, Provided (g/kg mixture): Lincomycin hydrochloride 22, Spectinomycin sulphate 22, with mineral oil USP 10 and soybean meal 946 as carrier. Supplied by the Upjohn Company, Animal Health Division, Orangeville, ON.

Table III-2. Partial chemical and amino acid composition (%)[a] of the experimental diets

Items	Diets	
	B+SBM	W+SBM
Dry matter	89.7	88.9
Organic matter	93.4	93.5
Crude protein	22.8	22.5
Gross energy (Mcal/kg)	4.4	4.4
Neutral-detergent fiber	9.3	9.8
Acid-detergent fiber	3.0	3.6
ß-glucans[b]	3.8	.8
Amino acids:		
Indispensable		
Arginine	1.31	1.25
Histidine	.54	.55
Isoleucine	1.02	.97
Leucine	1.64	1.61
Lysine	1.14	1.07
Phenylalanine	1.00	1.08
Threonine	.84	.80
Valine	1.15	1.08
Dispensable		
Alanine	.96	.93
Aspartic acid	2.22	2.16
Glutamic acid	4.69	5.13
Glycine	.87	.88
Serine	1.07	1.09
Tyrosine	.64	.58

[a] Dry matter basis.

[b] ß-glucan contents of barley, wheat and soybean meal were 5.80, 1.14 and .31%, respectively.

Table III-3. Effects of ß-glucanase supplementation on ileal and fecal digestibilities (%) of dry matter, organic matter, crude protein, gross energy, ß-glucans and amino acids in the B+SBM diet

Items	Ileal digestibilities			Fecal digestibilities		
	Control	ß-glucanase	SE[a]	Control	ß-glucanase	SE[a]
Dry matter	60.1[c]	66.7[b]	2.23	84.6[c]	86.4[b]	.45
Organic matter	63.0[c]	69.6[b]	2.14	87.0[c]	88.9[b]	.42
Crude protein	65.2[C]	73.5[B]	1.65	80.5[C]	85.9[B]	.84
Gross energy	64.9[c]	71.1[b]	1.89	84.6[C]	87.2[B]	.52
ß-glucans	80.1[C]	92.1[B]	2.65	99.4	99.5	.18
Amino acids:						
Indispensable						
Arginine	79.4[C]	84.3[B]	1.07	88.8[C]	91.6[B]	.52
Histidine	76.9[c]	81.9[b]	1.15	87.9[C]	91.0[B]	.53
Isoleucine	71.0[C]	77.6[B]	1.28	81.9[C]	86.2[B]	.85
Leucine	71.6[C]	78.3[B]	1.25	84.2[c]	87.6[b]	.70
Lysine	69.0	73.1	2.41	80.9[C]	85.5[B]	.85
Phenylalanine	74.1[C]	80.1[B]	.98	86.0[C]	89.0[B]	.55
Threonine	57.9[C]	67.7[B]	1.71	79.8[c]	84.7[b]	1.13
Valine	69.9[C]	76.7[B]	1.43	82.8[C]	86.9[B]	.82
Dispensable						
Alanine	61.8[C]	70.3[B]	1.66	78.7[c]	83.4[b]	1.00
Aspartic acid	70.4	73.9	1.37	85.3[c]	88.0[b]	.76
Glutamic acid	79.5	83.4	1.26	91.4[C]	93.5[B]	.40
Glycine	57.7	64.9	3.29	81.5[C]	85.4[B]	.84
Serine	68.9[C]	75.2[B]	1.35	86.2[c]	88.8[b]	.70
Tyrosine	66.7[c]	77.1[b]	2.70	81.5[C]	86.8[B]	.88

[a] Standard error of the mean (n = 6).

[b,c] Means in the same row, within ileal or fecal digestibilities, with different superscript letters differ ($P < .05$).

[B,C] Means in the same row, within ileal or fecal digestibilities, with different superscript letters differ ($P < .01$).

Table III-4. Effect of ß-glucanase supplementation on ileal and fecal digestibilities (%) of dry matter, organic matter, crude protein, gross energy, ß-glucans and amino acids in the W+SBM diet

Items	Ileal digestibilities			Fecal digestibilities		
	Control	ß-glucanase	SE[a]	Control	ß-glucanase	SE[a]
Dry matter	64.6	68.4	2.40	86.5	87.5	.35
Organic matter	68.3	71.5	2.12	88.9	89.8	.34
Crude protein	68.8	75.9	3.05	85.7	88.4	1.15
Gross energy	66.9	71.2	2.54	86.3[c]	87.9[b]	.41
ß-glucans	76.4[c]	86.8[b]	2.81	99.3	99.3	1.66
Amino acids:						
Indispensable						
Arginine	84.0	86.8	1.41	91.9	93.2	.64
Histidine	81.7	85.7	1.64	92.0	93.1	.68
Isoleucine	78.3	82.1	2.00	86.4	88.6	.96
Leucine	78.5	82.3	1.97	88.1	90.0	.74
Lysine	74.1	79.4	2.46	85.3	87.9	1.39
Phenylalanine	81.6	84.7	1.65	89.4	91.1	.62
Threonine	64.2	71.5	2.81	83.5	86.6	1.05
Valine	76.9	80.7	1.94	86.6	88.8	.95
Dispensable						
Alanine	67.8	74.6	3.02	83.4	86.1	1.42
Aspartic acid	75.6	81.3	1.45	87.9	90.1	1.03
Glutamic acid	87.1	89.8	1.39	94.3	95.3	.52
Glycine	64.2	70.4	3.54	85.7	88.0	.94
Serine	74.7	79.7	2.10	89.2	91.0	.64
Tyrosine	76.8	80.0	2.31	86.9	88.4	.97

[a] Standard error of the mean (n = 6) .

[b,c] Values in the same row, within ileal or fecal digestibilities, with different superscript letters differ ($P < .05$).

Table III-5. Effects of ß-glucanase supplementation on the net disappearance (g/kg DMI) of dry matter, organic matter, crude protein, gross energy, ß-glucans and amino acids in the large intestine of pigs fed the B+SBM and W+SBM diets

	B+SBM			W+SBM		
Items	Control	ß-glucanase	SE[a]	Control	ß-glucanase	SE[a]
Dry matter	267.8[b]	197.0[c]	14.96	220.1	190.6	23.32
Organic matter	245.6[b]	180.9[c]	11.60	194.4	170.3	19.46
Crude protein	33.7	28.5	4.99	38.0	28.2	6.73
Gross energy (Mcal)	1.0[b]	.8[c]	.07	.9	.7	.11
ß-glucans	7.3[b]	2.8[c]	.99	1.8	.9	.30
Amino acids						
Indispensable						
Arginine	1.06	.96	.12	1.04	.79	.17
Histidine	.53	.49	.06	.59	.41	.09
Isoleucine	.99	.87	.12	.83	.64	.19
Leucine	1.88	1.52	.18	1.60	1.23	.31
Lysine	1.14	1.04	.27	1.25	.91	.27
Phenylalanine	1.23	.99	.11	.88	.69	.17
Threonine	1.74	1.43	.19	1.54	1.22	.21
Valine	1.33	1.18	.17	1.09	.87	.20
Dispensable						
Alanine	1.56	1.26	.20	1.50	1.07	.27
Aspartic acid	3.40	3.11	.42	2.79	2.25	.48
Glutamic acid	5.78	4.75	.76	3.83	2.81	.81
Glycine	2.12	1.78	.33	1.91	1.54	.29
Serine	1.83	1.46	.19	1.63	1.22	.22
Tyrosine	.73	.63	.12	1.25	.49	.31

[a] Standard error of the mean (n = 6).

[b,c] Means in the same row, within diet, with different superscript letters differ ($P < .05$).

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CHAPTER IV

RESPONSE OF EXOCRINE PANCREATIC SECRETIONS TO FEEDING NUTRISOY VERSUS AUTOCLAVED NUTRISOY DIETS IN GROWING PIGS

A. Introduction

Since soybean trypsin inhibitors (SBTI) were first crystallized and their properties characterized (Bowman, 1944; Kunitz, 1945, 1946, 1947; Birk et al., 1963), their detrimental effects on animal nutrition and performance have been extensively studied. Feeding raw soybean or SBTI elicited adverse nutritional, biological and physiological responses in rats (Rackis, 1972; Rackis et al., 1979; Liener and Kakade, 1980), chickens (Chernick et al., 1948; Yen et al., 1973; Herkelman et al., 1993) and pigs (Yen et al., 1974; Cook et al., 1988). Soybean trypsin inhibitors inactivate trypsin (and also chymotrypsin) by the formation of an inhibitor-substrate complex (Blow et al., 1974; Sweet et al., 1974). Rats and chickens fed SBTI or raw soybean respond by hypersecretion of pancreatic enzymes and hypertrophy of the pancreas. Trypsin, which contains 8.7% cystine, accounts for half of the cystine secreted in pancreatic juice of rats fed raw soybean (Barnes et al., 1965a). Hypersecretion of pancreatic enzymes results in a higher demand for cystine by the pancreas, which may create a deficiency of the sulphur-containing amino acids (Barnes and Kwong, 1965; Barnes et al., 1965b).

There is a scarcity of information on the effect of feeding soybean products, high in SBTI, on the pancreatic secretions in pigs. Therefore, studies were carried out to determine the effect of feeding Nutrisoy (a food grade defatted soy flour; Archer Daniels Midland Company, Decatur, IL), which has a relatively high content of SBTI, and autoclaved Nutrisoy, which has a relatively low content of SBTI, on the pancreatic secretions in growing pigs prepared for total collection of pancreatic juice by the "Pouch" cannulation

technique (Hee et al., 1985).

B. Experimental Procedures

Animals and diets

Six PIC barrows (Camborough x Canabrid), average initial BW 27.2 ± 1.6 kg, were obtained from the University of Alberta Swine Research Unit. The barrows were housed individually in stainless steel metabolism crates (length: 140 cm; height: 85 cm; width: 80 cm) in a barn with automatic temperature control (25 ± 1°C) and given *ad libitum* access to a 16% CP grower diet (Sauer et al., 1983). Water was freely available from a low-pressure drinking nipple.

Nine days later, the barrows, average BW 31.5 ± 2.7 kg, were surgical fitted with pancreatic re-entrant cannulas for long term collection of pancreatic juice. Procedures for pre-operative care, surgery and post-operative care were carried out according to Hee et al. (1985). The pancreatic re-entrant cannula was constructed according to Hee et al. (1985) with modifications described by Ozimek et al. (1986). The barrows were immediately returned to the metabolism crates after surgery and fasted that same day. The next day they were given approximately 100 g of an 18% CP starter diet (Sauer et al., 1983), twice daily at 0830 and 2030. The feed allowance was gradually increased until the pigs consumed the diet at a daily rate of 5% (wt/wt) of BW. The barrows were allowed a 9-d recuperation period.

Two corn starch-based Nutrisoy model diets (Table IV-1) were formulated to contain 20% CP from either Nutrisoy or autoclaved Nutrisoy. The Nutrisoy (a food grade defatted soy flour) was provided by Archer Daniels Midland Company, Decatur, IL. The Nutrisoy was autoclaved with an Amsco 3201 Gravity Sterilizer (American Sterilizer Company, Erle, PA) using steam at 120°C, 32 psi, for 15 min followed by 5 min drying. Autoclaving reduced the content of SBTI in the Nutrisoy. Proximate analysis and amino acid compositions of Nutrisoy and autoclaved Nutrisoy are presented in Table IV-2. In this study both Nutrisoy and autoclaved Nutrisoy were used as ingredients to determine the effect of

dietary SBTI levels on pancreatic secretions. After autoclaving, the Nutrisoy turned into hard clumps and it was, therefore, ground through a 2-mm mesh screen before incorporation into the diet. Dextrose (10%) was included in the diet to improve palatability. Solkafloc (6%) and canola oil (5%) were also included in the diets. Vitamins and minerals were supplemented to meet or exceed NRC (1988) standards.

The experiment was carried out according to a two-period crossover design (Petersen, 1985). Each experimental period lasted nine days. The pigs were fed twice daily, equal amounts, at 0830 and 2030, at a daily rate of 5% (wt/wt) of BW which was determined at the beginning of each experimental period. The average BW at the start of the first and second experimental periods were 33.5 ± 2.7 and 37.2 ± 3.7 kg, respectively. The average BW at the conclusion of the experiment was 41.8 ± 3.9 kg. Pancreatic juice was collected for 24 h: from 0830 to 2030 on d 7 and from 2030 on d 8 to 0830 on d 9 of each experimental period. The collection, sampling and subsequent return of pancreatic juice was carried out according to Hee et al. (1985). Pancreatic juice was collected each h during the 12-h collection period. A sample of 10% (v/v) of the hourly volume was taken. The remainder of the pancreatic juice was made up to its original volume with saline and returned to the pig. The samples were frozen immediately after collection at -20° C. The experimental proposal and surgical procedures were reviewed and approved by the Animal Care Committee of the Faculty of Agriculture, Forestry, and Home Economics at the University of Alberta. The animals were cared for in accordance with the guidelines established by CCAC (1980).

Chemical and Statistical Analyses

Samples of the diets were taken each time the meal allowance was weighed out and finally pooled for each dietary treatment. Samples of diets were ground in a Wiley mill (Arther H. Thomas Co., Philadelphia, PA) through a .8-mm mesh screen before analysis. Analyses for DM, nitrogen and EE were carried out according to AOAC (1984). Analyses for NDF and ADF were carried out according to procedures outlined by Goering and van Soest (1970). The GE content was determined with an AC-300 Leco Automatic Calorimeter

(Leco Corporation, St. Joseph, MO). The procedures for amino acid analysis of the diets was previously described in Chapter III and Li and Sauer (1994). Tryptophan and proline were not measured.

Soybean trypsin inhibitor content in Nutrisoy and diets were determined according to procedures based on Hamerstrand et al. (1981) and Kakade et al. (1969) with modifications described in Appendix 3. The SBTI content in the sample was calculated based on the amount of trypsin units (TU) inhibited. One trypsin unit is arbitrarily defined as the increase of .01 absorbance units at 410 nm in 10 mL of incubation mixture; the trypsin inhibitor activity is defined as the amount of TU inhibited under the conditions defined (Kakade, et al., 1969).

Kakade et al. (1969), using purified trypsin, found that 1 μg "pure" trypsin has an activity of 1.9 TU which is equivalent to .019 absorbance units. For each μg pure trypsin inhibited, there will be a decrease of .019 absorbance units in the sample tested. Based on these findings, the SBTI content in the sample was calculated using the following formula:

SBTI (mg/g sample) = ($A_{standard}$ - A_{sample})/.019 x (1mg/1,000μg) x dilution factor/sample (g)

Where $A_{standard}$ = Absorbance of trypsin standards

A_{sample} = Absorbance of samples.

For nitrogen, protein content and enzyme activity determination, the hourly samples of pancreatic juice were thawed at 4°C and pooled within pig within each 12 h collection period. All analyses were carried out using the pooled samples. Total nitrogen in pancreatic juice was measured with a Leco FP-428 Nitrogen Analyzer (Leco Corporation, St. Joseph, MO). Pancreatic juice was weighed (100 mg) directly into an aluminium sample vial and loaded onto the automatic sampling rotator.

Protein content in pancreatic juice was determined according to the method of Lowry et al. (1951) using bovine serum albumin (Sigma Chemical Company, St. Louis, MO; Code: P0914) as standard. The standard curve of protein was set up by preparing a series of borosilicate glass tubes (13 x 100 mm) with increasing amounts of the standard. The preparation of standards was carried out under same conditions as for the pancreatic juice.

The pancreatic juice was thawed at 4° C and .6 mL juice was diluted with deionized water to 50 mL. Two test tubes for each sample were prepared. To each tube 1.2 mL of the diluted juice and 3.0 mL copper reagent were added and mixed thoroughly with Vortex Genie™. The mixture was kept at room temperature for 30 to 60 min before Folin color reagent (.3 mL/tube) was added. The tubes were allowed to stand for another 45 min at room temperature for the color to develop. The optical density was measured at 660 nm against deionized water as reference using a Milton Roy Spectronic 3000 Array Spectrophotometer equipped with auto-sampling pump (Fisher Scientific, Edmonton, AB, Canada)

The free amino acid content in pancreatic juice was determined as follows: vials containing 2 mL of pooled undiluted pancreatic juice were thawed at 4° C. The vials were placed in a rack in an ice bath and 500 µL of pancreatic juice from each vial were pipetted into 10 x 75 mm disposable borosilicate glass tubes; 100 µL of internal standard solution (340 µM ethanolamine hydrochloride and 320 µM DL-ß-amino-n-butyric acid dissolved in distilled and deionized water) was added to each tube. The contents were mixed using a Vortex Genie™ (Scientific Industries, Bohemia, NY); proteins were precipitated by adding 400 uL of 5% (w/v) trichloroacetic acid. The contents were mixed again several times and centrifuged at 1,100 x *g* at 4° C for 15 min to pellet the precipitated proteins. After centrifugation, 100 µL of the supernatant was used for amino acid analysis.

The total amino acid content in pancreatic juice was determined following acid hydrolysis. Undiluted pancreatic juice (500 µL) was pipetted into 13 x 100 mm screw-capped test tubes, mixed with 6 mL 6 M HCL, flushed with nitrogen gas, capped and hydrolyzed at 110°C for 24 h. Both total and free amino acids were analyzed according to Jones and Gilligan (1983) using a Varian 5000 high-performance liquid chromatography system (Varian Canada, Inc., Mississauga, ON). A detailed description of the procedure was presented in Chapter III.

Protein-bound amino acids were calculated as the difference between total and free amino acids. During hydrolysis, glutamine and asparagine are converted to glutamic and aspartic acid, respectively. Therefore, protein-bound glutamic and aspartic acid were calculated by subtracting free glutamic acid plus free glutamine and free aspartic acid plus

free asparagine from total glutamic and aspartic acid, respectively. The daily flows of total, protein-bound and free amino acids were calculated by multiplying the concentrations of total, protein-bound and free amino acids by the volume of pancreatic juice secreted per 24 h.

Amylase (EC 3.2.1.1) activity in pancreatic juice was determined according to procedures described by Rick and Stegbauer (1974) and the Enzyme Manual (WBC, 1988); lipase (EC 3.1.1.1) activity according to Schmidt et al. (1974). Activities of trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) in pancreatic juice were determined according to Rick (1974a,b) following activation of chymotrypsinogen and trypsinogen to chymotrypsin and trypsin, respectively, by enterokinase (Sigma; enteropeptidase, EC 3.4.21.9; Code: E0632). The activation procedure was carried out according to Glazer and Steer (1977) with modifications described by Gabert et al. (1995). Firstly, .2 mL undiluted pancreatic juice was added to 1.8 mL of buffer (pH 8.1) containing 100 µg/mL bovine serum albumin (Sigma; Code: A3059), 50 mM $CaCl_2$ and 50 mM Tris-[hydroxymethyl]-aminomethane (Sigma; Code: T1378). The enterokinase was dissolved in deionized water (10 mg/mL) and centrifuged at 15,600 x *g* at 5°C for 15 min to remove cell debris. Activation was initiated by adding .2 mL of diluted pancreatic juice to .2 mL supernatant which contained the enterokinase followed by incubation at 5° C for 3 and 120 h for chymotrypsin and trypsin, respectively.

The specific activity of enzymes in pancreatic juice is expressed as units (U) per litre (U x 10^{-3}/L); the total enzyme activity as U per 24 h (U x 10^{-3}/24 h). One U of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 µmol substrate in 1 min at 25°C. Total enzyme activities were calculated as the product of specific activity and the total volume of pancreatic juice secreted in 24 h. Nitrogen and protein contents were expressed as g/L. Since there are no differences ($P > .05$) in pancreatic secretions between day (0800 to 2000) and night (2000 to 0800) (Hee et al. 1988b), the total contents of nitrogen, protein and enzyme activities are presented on a 24-h rather than 12-h basis.

Data were subjected to analysis of variance according to procedures described by Steel and Torrie (1980) using the General Linear Model procedure of SAS (1990). The model

included dietary treatments, experimental periods and interactions between treatments and periods as sources of variation. The least square means of dietary treatments and experimental periods were compared with the Students' *t*-test (Steel and Torrie, 1980).

C. Results and Discussion

The pigs remained healthy and usually consumed their meal allowances within one hour after feeding. Postmortem examinations conducted at the conclusion of the experiment revealed no intestinal abnormalities and adhesions.

The chemical analyses and amino acid compositions of the experimental diets are presented in Table IV-2. The process of autoclaving did not affect the amino acid content. Autoclaving decreased the SBTI content in Nutrisoy from 38.5 to 9.3 mg/g. The SBTI contents in the diets containing Nutrisoy and autoclaved Nutrisoy were 13.3 and 3.3 mg/g, respectively.

The incorporation of Nutrisoy compared with autoclaved Nutrisoy into the corn starch-based diet increased ($P < .01$) the total volume of secretion of pancreatic juice (Table IV-3). There was a decrease ($P < .05$) in the concentration of nitrogen and protein in pancreatic juice in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. The results from these studies are in agreement with those reported by Zebrowska et al. (1985) who observed a decrease ($P < .05$) in volume of secretion of pancreatic juice when heat-treated (1,710 mL/24h) compared with raw soybean (2,908 mL/24h) was included in a corn starch-based diet. The aforementioned authors also found no effect ($P > .05$) on the daily nitrogen and protein secretions in pancreatic juice.

With respect to the enzyme activities in pancreatic juice, the inclusion of Nutrisoy compared with autoclaved Nutrisoy into the diet decreased the specific activity of amylase ($P < .01$), chymotrypsin ($P < .01$) and trypsin ($P < .05$), but not of lipase (Table IV-3). There was no effect ($P > .05$) on the total activities of the enzymes. These results are in agreement with studies reported by Zebrowska et al. (1985) who observed no effect ($P > .05$) on the total activities of trypsin, chymotrypsin and amylase when heat-treated compared

with raw soybean meal was included in a corn starch-based diet.

These studies with pigs showed no effect of SBTI on the total activities of the exocrine pancreatic enzymes. Studies with chicks and rats fed SBTI (or raw versus heat-treated soybean meal) have shown an increase in pancreatic enzyme secretions and also hypertrophy of the pancreas (Liener and Kakade, 1980). In these species, according to the negative feedback regulation mechanism proposed by Green and Lyman (1972), trypsin (and also chymotrypsin) are inactivated by the formation of an inhibitor-substrate complex. Finally, the inhibition of trypsin and chymotrypsin activities will trigger an increased secretion of cholecystokinin which stimulates the secretion of pancreatic enzymes. In this context, some researchers (Goss, 1966; Schingoethe et al., 1970) suggested that there was a direct relationship between the size of the pancreas, in relation to BW, and the sensitivity of response to SBTI or raw soybean. They postulated that species of animals with a pancreas weight exceeding .3% of BW (which include rats and chicks) are more prone to pancreatic hypersecretion and hypertrophy than species in which the ratio of pancreas to BW is less than .3%. This, in turn, may affect the requirement for the sulphur-containing amino acids. The exocrine pancreatic enzymes are rich in cystine. Species with a relatively large pancreas, which is positively correlated with pancreatic enzyme secretion, will therefore have a relatively higher requirement of the sulphur-containing amino acids when fed SBTI or raw soybean.

The effect of experimental period on the parameters measured is presented in Table IV-4. The average BW of the pigs were 35.4 and 39.5 kg during periods 1 and 2, respectively. The total volume of secretion and nitrogen and protein contents were higher ($P < .05$) during period 2. There were no differences ($P > .05$) in the concentrations of nitrogen and protein in pancreatic juice between experimental periods. With respect to the pancreatic enzymes, both specific ($P < .01$) and total activities ($P < .05$) of amylase were higher during period 2. As well, the total activity of chymotrypsin was higher ($P < .05$) during period 2. These results confirm those of Weström et al. (1988); as BW increases secretion rate increases. Gabert et al. (1995) also reported an increase in secretion volume, protein content and total activities of some of the exocrine pancreatic enzymes between

periods in the same experiment or between experiments. Some other studies (e.g., Mosenthin and Sauer, 1991) did not show a period effect. In all likelihood, a period effect will only be observed if there is a certain increase in BW from one experimental period to the next.

As was previously discussed by Hee et al. (1985, 1988a,b), it is essential to consider total rather than specific activities of enzymes when studies are carried out to determine the effect of dietary treatment on pancreatic enzyme secretions. To illustrate this point, one can, for example, refer to studies by Yen et al. (1977) with pigs fed raw soybean compared with solvent-extracted soybean meal diets. The slaughter method was used to determine the activities of trypsin and chymotrypsin in digesta from the small intestine. The activities of these enzymes were lower ($P < .05$) when raw soybean was fed. These lower activities may have resulted from a lower pancreatic secretion and/or a dilution effect since there is a larger volume of digesta in the small intestine when raw soybean rather than heat-treated soybean meal is fed. To answer this question, it is necessary to determine the total secretions (activities) of the exocrine pancreatic enzymes. The total enzyme activities in pancreatic juice can be determined with either the direct pancreatic cannulation (Corring, 1975) or the "Pouch" technique (Hee et al., 1985). The "Pouch" technique was chosen for this study, as this method allows for both long term and total collection of pancreatic juice.

In the interpretation of results from these studies, little attempt has been made to compare these with those reported in the literature, foremost perhaps because of differences in BW between pigs. As was pointed out by Imbeah et al. (1988), a direct comparison of results, as opposed to relative differences, resulting from various dietary treatments, is very difficult for reasons of differences in feed intake, feeding regimen, diet composition, BW, and procedures for enzyme analysis and techniques used to collect pancreatic juice.

The concentration (mM/L) of each amino acid, in the total, free and protein-bound amino acid pools, was higher in pancreatic juice collected from pigs fed autoclaved Nutrisoy (Table IV-5), which corresponds to the higher protein and enzyme contents (Table IV-3). In the free amino acid pool, of the indispensable amino acids, the highest concentrations were observed for leucine and lysine. In the protein-bound pool, the highest concentrations were found for aspartic acid, cysteine, glutamic acid, glycine and serine. The high levels of

these protein-bound amino acids reflect their relatively high contents in the pancreatic enzymes. For example, chymotrypsin contains high levels of aspartic acid, glycine and alanine (Charles et al., 1967), while trypsin contains high levels of aspartic acid, glutamic acid, glycine and serine (Charles et al., 1963). Amylase and lipase also contain relatively high levels of some of the aforementioned amino acids (Cozzone et al., 1970; Winkler et al., 1990).

The effect of experimental diet on the total amino acid secretion (mM/24 h) in pancreatic juice is presented in Table IV-6. The increase in the total secretion of each of the amino acids reflects the increase in protein secretion when autoclaved Nutrisoy rather than Nutrisoy is fed (Table IV-3). For most of the amino acids in the free and protein-bound amino acid pools, the increase is significant ($P < .05$). As these studies show, the free amino acid pool in pancreatic juice makes up a substantial proportion of the total amino acid pool; from 1.3 (aspartic acid) to 36.8% (leucine) in pigs fed the autoclaved Nutrisoy diet and from 1.4 (aspartic acid) to 30.6% (leucine) in pigs fed the Nutrisoy diet. Furthermore, the content of the indispensable amino acids is relatively high in the free amino acid pool. On the other hand, the content of the dispensable amino acids is relatively high in the protein-bound amino acid pool.

In conclusion, feeding diets containing high and low levels of SBTI (simulated by feeding Nutrisoy versus autoclaved Nutrisoy) to pigs did not affect ($P > .05$) the total activities of the exocrine pancreatic enzymes. Therefore, the detrimental effect of SBTI does not appear to be the result of hypersecretion of pancreatic enzymes per sé. Other mechanisms, for example, formation of enzyme-inhibitor complexes, may be responsible for the detrimental effect. Further studies, presented in Chapter V, were carried out to determine the effect of SBTI on amino acid and GE digestibilities.

D. Implications

The feeding of the corn starch-based Nutrisoy compared with the autoclaved Nutrisoy diet to growing pigs increased the volume of secretion of pancreatic juice. There

was no effect of dietary treatment on the total activities of exocrine pancreatic enzymes. The negative feedback regulation mechanism that stimulates compensatary secretion of pancreatic trypsin and chymotrypsin following the feeding of diets high in SBTI content, which is observed in chicks and rats, is absent in growing pigs.

Table IV-1. Formulation of the Nutrisoy and autoclaved Nutrisoy diets (%)

Ingredients	Diets	
	Nutrisoy	Autoclaved Nutrisoy
Corn starch	40.0	40.0
Nutrisoy[a]	35.0	-
Nutrisoy (autoclaved)	-	35.0
Canola oil	5.0	5.0
Dextrose	10.0	10.0
Solkafloc	6.0	6.0
Iodized salt[b]	.3	.3
Mineral-vitamin premix[c]	1.0	1.0
Biophos[d]	1.6	1.6
Calcium Carbonate[e]	.5	.5
Chromic oxide	.3	.3
Antibiotics[f]	.15	.15
DL-Methionine	.15	.15

[a] Defatted soy flour. Supplied by Archer Daniels Midland Company, Decatur, IL.

[b] Provided (%): NaCL, 99 and I, .015. Supplied by Sift Canada Inc., Mississauga, ON.

[c]. Provided the following (per kg diet): Vitamin A, 7,500 IU; vitamin D_3, 500 IU; vitamin E, 40 IU; vitamin K_3, 2 mg; vitamin B_{12}, .03 mg; riboflavin, 12 mg; niacin, 40 mg; pantothenic acid, 25 mg; choline, 600 mg; biotin, 0.25 mg; folic acid, 1.6 mg; thiamin, 3.0 mg; Ethoxyquin, 5 mg; Fe, 150 mg; Mn, 20 mg; Zn, 120 mg; Cu, 125 mg; I, .2 mg; Se, .3 mg. Supplied by Hoffmann-LaRoche Ltd., 2455 Meadowprine Blvd., Mississauga, ON

[d] Provided (%): Available phosphorous, 15-18; calcium, 24. Supplied by Continental Lime Ltd. Exshaw, AB.

[e] Provided (%): Calcium, 38. Supplied by Continental Lime Ltd. Exshaw, AB.

[f] Veterinary LS-20 premix, provided the following (g/kg mixture): Lincomycin hydrochloride, 22, and Spectinomycin sulphate, 22 with mineral oil USP 10, and soybean meal, 946 as carrier. Supplied by the Upjohn Company, Animal Health Division, Orangeville, ON.

Table IV-2. Proximate analyses and amino acid composition (%)[a] of Nutrisoy, autoclaved Nutrisoy and the experimental diets

Items	Nutrisoy	Autoclaved Nutrisoy	Nutrisoy diet	Autoclaved Nutrisoy diet
Dry matter	92.6	92.6	91.9	91.7
Crude protein	57.5	57.2	20.3	20.7
Gross energy (Mcal/kg)	4.8	4.8	4.5	4.5
Neutral-detergent fiber	7.3	7.5	8.0	8.0
Acid-detergent fiber	4.0	4.1	6.6	6.6
Ether extract	1.2	.9	6.1	5.9
SBTI (mg/g)	38.4	9.3	13.3	3.3
Amino acids:				
Indispensable				
Arginine	3.80	3.70	1.38	1.39
Histidine	1.36	1.33	.50	.50
Isoleucine	2.46	2.45	.91	.94
Leucine	3.99	3.98	1.49	1.55
Lysine	3.35	3.09	1.21	1.17
Methionine	.93	.96	.77	.77
Phenylalanine	2.66	2.65	1.00	1.04
Threonine	1.91	1.90	.72	.73
Valine	2.44	2.44	.91	.95
Dispensable				
Alanine	2.24	2.24	.85	.86
Aspartic acid	5.17	5.13	1.96	1.97
Cysteine	.86	.95	1.00	.86
Glutamic acid	9.17	9.09	3.49	3.62
Glycine	2.24	2.24	.84	.86
Serine	2.47	2.46	.94	.96
Tyrosine	1.53	1.56	.52	.55

[a] Dry matter basis.

Table IV-3. Effect of experimental diet on the secretion volume, nitrogen and protein contents and enzyme activities of pancreatic juice collected from growing pigs

Diets		Nutrisoy	Autoclaved Nutrisoy	SE[a]
Volume	mL/24 h	3803.6[D]	2633.1[E]	104.38
Nitrogen[b]	g/L	1.3[e]	2.0[d]	.08
	g/24 h	5.0	5.2	.16
Protein[c]	g/L	6.2[e]	10.3[d]	.58
	g/24 h	22.8[e]	25.7[d]	1.26
Enzyme activities:				
Amylase	$Ux10^{-3}$/L	107.4[E]	159.5[D]	.61
	$Ux10^{-3}$/24h	414.3	420.8	18.68
Lipase	$Ux10^{-3}$/L	32.7	36.3	2.07
	$Ux10^{-3}$/24h	108.1	95.6	9.22
Chymotrypsin	$Ux10^{-3}$/L	42.1[E]	60.3[D]	1.38
	$Ux10^{-3}$/24h	139.0	154.0	6.96
Trypsin	$Ux10^{-3}$/L	46.0[e]	73.5[d]	6.62
	$Ux10^{-3}$/24h	168.5	178.9	10.60

[a] Standard error of the mean (n = 10).
[b] Determined as total nitrogen based on the Kjeldahl procedure.
[c] Determined according to Lowry et al. (1951).
[d,e] Means in the same row with different superscript letters differ ($P < .05$).
[D,E] Means in the same row with different superscript letters differ ($P < .01$).

Table IV-4. Effect of experimental period on the secretion volume, nitrogen and protein contents and enzyme activities of pancreatic juice collected from growing pigs

Items		Experimental Period 1	2	SE[a]
Volume	mL/24 h	2731.6[c]	3505.1[b]	104.38
Nitrogen	g/L	1.7	1.7	.08
	g/24 h	4.9[c]	5.8[b]	.16
Protein	g/L	7.9	8.6	.58
	g/24 h	21.4[c]	27.4[b]	1.26
Enzyme activities:				
Amylase	$Ux10^{-3}$/L	130.4[C]	136.5[B]	.61
	$Ux10^{-3}$/24h	371.6[c]	463.6[b]	18.68
Lipase	$Ux10^{-3}$/L	38.1	30.9	2.07
	$Ux10^{-3}$/24h	106.5	103.9	9.24
Chymotrypsin	$Ux10^{-3}$/L	49.8	52.6	1.38
	$Ux10^{-3}$/24h	138.6[c]	171.6[b]	6.96
Trypsin	$Ux10^{-3}$/L	66.4	53.1	6.62
	$Ux10^{-3}$/24h	179.6	167.8	10.60

[a] Standard error of the mean (n = 10).
[b,c] Means in the same row with different superscript letters differ ($P < .05$).
[B,C] Means in the same row with different superscript letters differ ($P < .01$).

Table IV-5. Effect of experimental diet on amino acid concentration (mM/L) in pancreatic juice collected from growing pigs

	Total amino acids			Free amino acids			Protein-bound amino acids		
	Nutrisoy	Autoclaved Nutrisoy	SE[a]	Nutrisoy	Autoclaved Nutrisoy	SE[a]	Nutrisoy	Autoclaved Nutrisoy	SE[a]
Amino acids									
Indispensable									
Arginine	1.9[c]	3.5[b]	.30	.6	1.2	.17	1.3[c]	2.3[b]	.18
Histidine	1.0[c]	1.7[b]	.17	.2[c]	.5[b]	.05	.7	1.3	.14
Isoleucine	2.7[c]	4.6[b]	.37	.6[c]	1.2[b]	.12	2.0[c]	3.4[b]	.29
Leucine	3.7[c]	6.2[b]	.55	1.2[c]	2.4[b]	.24	2.5[c]	3.8[b]	.45
Lysine	2.3[c]	4.6[b]	.49	.8	2.1	.31	1.5[c]	2.5[b]	.35
Methionine	2.5[c]	3.1[b]	.74	.1[c]	.3[b]	.04	2.3	2.8	.76
Phenylalanine	1.8[c]	3.2[b]	.29	.5[c]	1.1[b]	.13	1.3	2.1	.20
Threonine	2.7[c]	4.8[b]	.43	.4	.8	.09	2.3[c]	4.1[b]	.37
Valine	3.5[c]	6.2[b]	.49	.5	1.1	.13	3.0[c]	5.1[b]	.40
Subtotal	22.1	37.9		4.9	11.8		16.9	27.4	
Dispensable									
Alanine	3.6[c]	6.2[b]	.55	.7	1.4	.18	2.9	4.8	.42
Aspartic acid	5.6[c]	9.3[b]	.62	.1	.2	.03	5.2[c]	8.5[b]	.55
Asparagine				.1	.2	.04			
Cysteine	4.8[c]	6.1[b]	.28	.5	.3	.28	4.3[c]	5.8[b]	.39
Glutamic acid	4.3[c]	7.4[b]	.63	.3[c]	.7[b]	.07	3.7[c]	6.2[b]	.55
Glutamine				.5[c]	1.0[b]	.10			
Glycine	5.1[c]	9.3[b]	.78	.3	.6	.09	4.9[c]	8.7[b]	.71
Serine	3.9[c]	6.8[b]	.57	.4	.9	.13	3.5[c]	5.9[b]	.47
Tyrosine	1.9[c]	3.3[b]	.26	.6	1.3	.16	1.3	2.0	.18
Subtotal	29.2	48.4		3.5	6.6		25.8	41.9	
Total	51.3	86.3		8.4	18.4		42.7	69.3	

[a] Standard error of the mean (n = 10).

[b, c] Values in the same row, within total, free and protein-bound amino acids, with different superscript letters differ ($P < .05$).

Table IV-6. Effect of experimental diet on total amino acid secretion (mM/24 h) in pancreatic juice collected from growing pigs

	Total amino acids			Free amino acids			Protein bound amino acids		
	Nutrisoy	Autoclaved Nutrisoy	SE[a]	Nutrisoy	Autoclaved Nutrisoy	SE[a]	Nutrisoy	Autoclaved Nutrisoy	SE[a]
Amino acids									
Indispensable									
Arginine	7.3[c]	8.6[b]	.27	2.5	2.8	.34	4.9[c]	5.8[b]	.39
Histidine	3.6[c]	4.2[b]	.12	.8[c]	1.1[b]	.04	2.9	3.2	.15
Isoleucine •	10.0[c]	11.3[b]	.19	2.1[c]	2.8[b]	.13	7.9[c]	8.5[b]	.22
Leucine	13.7[c]	15.2[b]	.39	4.2[c]	5.6[b]	.24	9.5	9.5	.50
Lysine	8.7[c]	11.0[b]	.38	2.9[c]	4.7[b]	.28	6.2	6.4	.30
Methionine	8.4	9.2	.65	.3	.8	.04	8.1	8.4	.63
Phenylalanine	6.7[c]	7.9[b]	.25	1.7[c]	2.5[b]	.13	5.0	5.4	.22
Threonine	10.1[c]	11.7[b]	.26	1.2[c]	1.8[b]	.06	8.9[c]	10.0[b]	.24
Valine	13.3[c]	15.2[b]	.27	1.9[c]	2.5[b]	.10	11.4[c]	12.6[b]	.25
Subtotal	81.8	94.3		17.6	24.6		64.8	69.8	
Dispensable									
Alanine	13.6[c]	15.3[b]	.37	2.3[c]	3.2[b]	.15	11.3	12.1	.32
Aspartic acid	21.0[C]	23.2[B]	.17	.3	.3	.05	19.6[c]	21.2[b]	.19
Asparagine				.3	.4	.04			
Cysteine	18.4	15.7	1.34	1.8	2.2	.08	16.3	13.9	1.79
Glutamic acid	16.1[c]	18.1[b]	.38	1.1[c]	1.6[b]	.08	14.1	15.4	.39
Glutamine				1.8[c]	2.4[b]	.11			
Glycine	19.5[c]	22.8[b]	.57	.9	1.3	.10	18.5[c]	21.5[b]	.48
Serine	14.9[c]	16.8[b]	.39	1.5	2.1	.13	13.4	14.7	.32
Tyrosine	7.3[c]	8.1[b]	.16	2.1[c]	2.8[b]	.14	5.2	5.2	.17
Subtotal	110.8	120.1		12.1	16.3		98.4	104.0	
Total	192.6	214.4		29.7	40.9		163.2	173.8	

[a] Standard error of the mean (n = 10).

[b,c] Values in the same row, within total, free and protein-bound amino acids, with different superscript letters differ ($P < .05$).

[B,C] Values in the same row, within total, free and protein-bound amino acids, with different superscript letters differ ($P < .01$).

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CHAPTER V

RESPONSE OF NUTRIENT DIGESTIBILITIES TO FEEDING NUTRISOY VERSUS AUTOCLAVED NUTRISOY DIETS IN GROWING PIGS

A. Introduction

Feeding raw soybean or soybean trypsin inhibitors (SBTI) to chicks (Chernick et al., 1948; Yen et al., 1973; Herkelman et al., 1993) and rats (Rackis, 1972; Rackis et al., 1979; Liener and Kakade, 1980) causes hypertrophy of the pancreas, hypersecretion of pancreatic enzymes and a reduction in nutrient digestibilities. Studies with pigs fed raw soybean did not show pancreatic hypertrophy (Yen et al., 1977) and hypersecretion of pancreatic enzymes (Chapter IV). Studies presented in Chapter IV showed that hypersecretion of pancreatic juice occurred only in terms of volume, but not in total enzyme activities. The results presented in Chapter IV suggest the absence of a negative feedback regulation mechanism for pancreatic enzyme secretion in growing pigs fed soy products containing high levels of SBTI. A decrease in performance (e.g., Yen et al., 1974; Cook et al., 1988;) and a decrease ($P < .05$) in fecal CP and energy digestibilities was also observed in pigs fed raw soybean (e.g., Combs et al., 1967). The detrimental effect of raw soybean or soy products that contain high level of SBTI in pigs may be due to the inhibition of enzyme activities by the formation of enzyme-inhibitor complexes. To further study the effect of SBTI on nutrient digestion and absorption, experiments were carried out to determine the effect of feeding Nutrisoy versus autoclaved Nutrisoy (a food grade defatted soy flour; Archer Daniels Midland Company, Decatur, IL), which contain relatively high and low levels of SBTI respectively, on the ileal as well as fecal digestibilities of energy, CP and amino acids in growing pigs.

B. Experimental Procedures

Animals and diets

Six PIC barrows (Camborough x Canabrid), average initial BW 47.8 ± 4.0 kg, were obtained from the University of Alberta Swine Research Unit. The barrows were housed individually in stainless steel metabolism crates (length: 140 cm; height: 85 cm; width: 80 cm) in a temperature-controlled barn (25 ± 1° C). The pigs were given *ad libtium* access to a 16% CP grower diet (Sauer et al., 1983); water was freely available from a low-pressure drinking nipple.

Four days later, the barrows were fitted with a simple T-cannula at the distal ileum according to procedures adapted from Sauer (1976). The cannulas were modified according to de Lange et al. (1989). The barrows were returned to the metabolism crates after surgery and fasted that same day. They were given approximately 100 g of an 18% CP starter diet (Sauer et al., 1983) the next day. The feed allowance was gradually increased until the pigs consumed the diet at a daily rate of 4% (wt/wt) of BW of each pig. The barrows were allowed a 9-d recuperation period. A detailed description of pre- and post-operative care was previously provided by Li et al. (1993).

Two corn starch-based diets were formulated to contain 20% CP from either Nutrisoy or autoclaved Nutrisoy (Table V-1). The preparation of the autoclaved Nutrisoy and formulation of the diets were previously described (Chapter IV). Chemical characterization of Nutrisoy, autoclaved Nutrisoy, and the experimental diets are presented in Table V-2.

The experiment was carried out according to a two-period crossover design (Petersen, 1985). Each experimental period lasted 12 d. The pigs were fed twice daily, equal amounts, at 0800 and 2000, at a daily rate of 4% (wt/wt) of BW which was determined at the beginning of each experimental period. The average BW at the start of the first and second experimental periods were 53.3 ± 3.7 and 61.0 ± 5.1 kg, respectively. The average BW at the conclusion of the experiment was 71.8 ± 7.6 kg. The ADG and FE were determined for each pig during each experimental period. The collection of feces was

initiated at 0800 on d 8 of each experimental period and continued for 48 h. Ileal digesta were collected for total of 24 h: from 0800 to 2000 on d 10 and from 2000 on d 11 to 0800 on d 12. Following the conclusion of each experimental period, an additional two days were used to carry out another study to determine the endogenous protein (amino acid) recoveries at the distal ileum using homoarginine as an internal marker according to Hagemeister and Erbersdobler (1985). The results from this study will be reported elsewhere.

The procedure for the collection of digesta and feces was previously described by Li et al. (1994). Feces and digesta were frozen at -20° C immediately after collection. The samples were pooled, leaving one sample of feces and digesta for each pig for each experimental period. The experimental proposal and surgical procedures were reviewed and approved by the Animal Care Committee of the Faculty of Agriculture, Forestry and Home Economics at the University of Alberta. The animals were cared for in accordance with the guidelines established by CCAC (1980).

Chemical and Statistical Analyses

Samples of digesta and feces were freeze-dried and ground in a Wiley mill through a .8-mm mesh screen before analysis. Analyses for DM, nitrogen, chromic oxide and amino acids in digesta and feces were performed according to procedures previously described in Chapter III.

Data were subjected to analysis of variance using the General Linear Model procedure of SAS (1990). The statistical model included dietary treatments, experimental periods and the interactions of treatment and period as sources of variation. Least square means were compared using the Students' *t*-test according to Steel and Torrie (1980). One of the pigs (pig #2, experimental period 2) partially rejected the diet containing Nutrisoy. Therefore, the results from this pig were not included in the statistical analysis.

C. Results and Discussion

With the exception of one pig that partially rejected the Nutrisoy diet, all other pigs consumed their meal allowances within 30 min after feeding throughout the experiment. Postmortem examinations carried out at the conclusion of the experiment showed no intestinal adhesions or other abnormalities.

The incorporation of autoclaved Nutrisoy compared with Nutrisoy into the corn starch-based diet increased ($P < .01$) the apparent ileal digestibilities of DM, OM, GE, CP and all amino acids (Table V-3). The GE digestibility increased from 67.1 to 78.0%; the CP digestibility from 41.2 to 77.6%. Of the indispensable amino acids, the increases ranged from 19.4 (methionine) to 46.4 (leucine) percentage units. Of the dispensable amino acids, the increases ranged from 27.7 (aspartic acid) to 46.9 (tyrosine) percentage units.

With the exception of methionine, the apparent ileal digestibilities of the indispensable amino acids in the Nutrisoy diet were uniformly low, ranging from 40.6 (leucine) to 48.7% (arginine). The apparent ileal digestibility of methionine is not reflective of Nutrisoy, as this amino acid was supplemented to the diet (Table V-1). These results are different from studies with other protein supplements. For example, in soybean meal, the apparent ileal digestibilities of arginine and lysine are relatively high whereas that of threonine is relatively low (Li and Sauer, 1994). As was discussed by Low (1980), of the indispensable amino acids, arginine and lysine would be expected to appear first after enzymatic hydrolysis and threonine last based on the known specificity of the proteases and peptidases involved. Indirectly, these results suggest that the formation of a complex between SBTI and trypsin (also chymotrypsin) may have resulted in an overall decrease in the efficiency of protein digestion due to loss of enzyme specificity. The enzyme-inhibitor complex is very strong due to the close complementary fit of these two interacting molecules; the binding force is further reinforced by a large number of non-covalent hydrophobic and hydrogen bonds (Blow et al., 1974; Sweet et al., 1974).

The incorporation of autoclaved Nutrisoy compared with Nutrisoy also increased ($P < .01$) the apparent fecal digestibilities of all parameters measured (Table V-3). The GE

digestibility increased from 88.3 to 90.8%; the CP digestibility from 79.1 to 90.1%. Of the indispensable amino acids, the increases ranged from 8.1 (histidine) to 14.9 (valine) percentage units. Of the dispensable amino acids, the increases ranged from 7.4 (cysteine) to 15.4 (tyrosine) percentage units.

As was pointed out previously (e.g., Knabe et al., 1989), the ileal rather than fecal analysis method should be used to determine amino acid digestibilities in feedstuffs for pigs because of the modifying action of the microflora in the large intestine. The differences in amino acid digestibilities between dietary treatments measured with the ileal analysis method were considerably larger than those measured with the fecal analysis method (Table V-3), which shows that the ileal analysis method is more sensitive than the fecal analysis method for detecting differences in amino acid digestibilities. On the other hand, the digestibility of GE should be determined with fecal analysis method, as there can be considerable disappearance of energy in the large intestine in the form of volatile fatty acids; the extent of this disappearance is dependent on diet composition (e.g., Fan et al., 1994).

Compared with pigs fed autoclaved Nutrisoy, pigs fed Nutrisoy had a lower ADG and FE. The ADG for pigs fed the Nutrisoy and autoclaved Nutrisoy diets were 523.2 and 795.3 g ($P < .01$), respectively. In the same order, the feed/gain ratios were 4.98 and 2.77 ($P < .01$), respectively (data not shown). Some caution should be exercised in the interpretation of the performance results as these are only based on a 12-d measurement period. However, the differences in performance reflect the differences in GE and amino acid digestibilities between dietary treatments.

The results of these studies are in general agreement with those reported in the literature in which diets containing raw soybean, which are high in SBTI, were evaluated. A decrease in performance, nitrogen retention and nutrient digestibilities were reported in studies with young (Yen et al., 1974; Cook et al., 1988), growing (Combs et al., 1967) and growing-finishing pigs (Jimenez et al., 1963; Young, 1967) when raw soybean diets were fed.

There was a considerably higher ($P < .01$) net disappearance (g/kg DMI) of DM, OM, CP, GE, and all amino acids in the large intestine of pigs fed Nutrisoy diet (Table V-4).

For CP, the net disappearance in the large intestine was 51.0 g higher; for GE, the net disappearance was .4 Mcal higher in pigs fed the Nutrisoy diet compared with the autoclaved Nutrisoy diet. For the indispensable amino acids, the differences of net disappearance between diets ranged from 1.2 (histidine) to 4.8 (leucine); for the dispensable amino acids from 1.7 (alanine and tyrosine) to 8.7 (glutamic acid). The pattern of amino acids disappearence in the large intestine was similar to but greater in magnitude than values reported in previous studies (e.g., Li and Sauer, 1994).

The disappeared amino acids in the large intestine of pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet will not contribute to the protein status of the pigs. As was originally pointed out by Zebrowska et al. (1973), microbial fermentation of undigested protein (and unabsorbed amino acids) entering the large intestine will yield nitrogen-containing compounds, mainly in the form of ammonia. Once absorbed, ammonia will be converted to urea and excreted in urine. On the other hand, the higher disappearance of GE in the large intestine in pigs fed the Nutrisoy diet way be partialy of benefit. Since microbial fermentation of undigested energy sources entering the large intestine will yield volatile fatty acids, once absorbed, these volatile fatty acids will be used by the pigs as supplementary energy sources. In terms of energy utilization, these results indicate a compensatary effect by the microflora in the large intestine. This compensatary effect will be important in pigs fed diets with a high content of non-starch polysaccharides, in particular when enenrgy supply in the diet becomes a limiting factor.

With the exception of valine, aspartic and glutamic acid, there were no differences ($P > .01$) in the apparent ileal digestibilities of the parameters that were measured between experimental periods 1 and 2 (Table V-5). These results are in agreement with previous studies with pigs in the same BW range in which there were usually no period effects on apparent ileal digestibilities (e.g., Fan et al., 1994). On the other hand, the apparent fecal digestibilities of all parameters measured were higher ($P < .01$) in period 2 than 1. The digestibility of CP increased by 4.6 percentage units. Of the amino acids, the increases ranged from 5.0 (arginine) to 8.8 (methionine) percentage units for the indispensable and from 3.9 (glutamic acid) to 10.0 (cysteine) percentage units for the dispensable amino acids.

These results are in contrast to previous studies (e.g., Fan et al., 1994) in which there was usually no effect of BW on ileal and fecal nutrient digestibilities. One can only speculate that the increase in fecal nutrient digestibilities with BW may have resulted from a further increase in the activity of the microflora in the large intestine following long-term feeding of Nutrisoy, irrespective if fed in raw or autoclaved form.

As was shown in Chapter IV, the feeding of the Nutrisoy compared with the autoclaved Nutrisoy did not affect ($P > .05$) the total activities of the exocrine pancreatic enzymes in growing pigs, indicating the absence of compensatory hypersecretion of pancreatic enzymes which is observed in chicks and rats. The considerable reduction in ileal amino acid and energy digestibilities upon feeding SBTI is likely caused by the formation of complexes between SBTI and trypsin and chymotrypsin. The increased losses of protein, therefore, are both of endogenous and exogenous origin. The increased endogenous losses result from the formation of trypsin and chymotrypsin complexes with SBTI, while the increased exogenous losses result from a decrease in the digestion of exogenous protein due to a decrease in available enzyme supply. Barth et al. (1993), using homoarginine as an internal marker, determined the endogenous protein losses at the distal ileum in miniature pigs fed raw and heat-treated soybeans. These authors reported endogenous and exogenous protein losses of 221 and 19 mM/test meal, respectively, in pigs fed heat-treated soybeans. In the same order, these losses were 432 and 40 mM/test meal in pigs fed raw soybeans. Barth et al. (1993), therefore, concluded that dietary SBTI affected protein digestibility more by increased losses of amino acids of endogenous rather than of exogenous origin. It is rather difficult to visualize the high endogenous losses reported by Barth et al. (1993). The total protein recoveries in ileal digesta in this study, expressed as g/24h per kg BW, were 3.92 and 1.49 for pigs fed the Nutrisoy and the autoclaved Nutrisoy diet, respectively. The total secretions of pancreatic protein, also expressed as g/24h per kg BW, were .80 and .62 for pigs fed the Nutrisoy and the autoclaved Nutrisoy diet, respectively (Chapter IV). Even in case that all pancreatic protein secreted in pigs fed the Nutrisoy diet would form complexes with SBTI, which is unlikely, only 20.3% of protein in ileal digesta would be of pancreatic origin.

In conclusion, the ileal as well as fecal digestibilities of GE and amino acids were considerably lower in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. The lower digestibilities are likely due to the formation of complexes between SBTI and trypsin and chymotrypsin and thus a reduction in available supply of these enzymes. Concomitantly with a decrease in the ileal GE and amino acid digestibilities, there was an increase in the net disappearance of GE and amino acids in the large intestine in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. Further studies are warranted to determine the endogenous amino acid losses in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet, particularly the extent of losses associated with the enzyme-inhibitor complex.

D. Implications

The ileal amino acid digestibilities were considerably lower in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. The lower amino acid digestibilities resulted from a decrease in the efficiency of protein digestion due to inactivation of digestive enzymes by SBTI and not from hypersecretion of the pancreatic enzymes.

Table V-1. Formulation of the Nutrisoy and autoclaved Nutrisoy diets (%)

Ingredients	Diets	
	Nutrisoy	Autoclaved Nutrisoy
Corn starch	40.0	40.0
Nutrisoy[a] (raw)	35.0	-
Nutrisoy (autoclaved)	-	35.0
Canola oil	5.0	5.0
Dextrose	10.0	10.0
Solkafloc	6.0	6.0
Iodized salt[b]	.3	.3
Mineral-vitamin premix[c]	1.0	1.0
Biophos[d]	1.6	1.6
Calcium Carbonate[e]	.5	.5
Chromic oxide	.3	.3
Antibiotics[f]	.15	.15
DL-Methionine	.15	.15

[a] Defatted soy flour. Supplied by Archer Daniels Midland Company, Decatur, IL.

[b] Provided (%): NaCL, 99 and I, .015. Supplied by Sift Canada Inc., Mississauga, ON.

[c] Provided the following (per kg diet): Vitamin A, 7,500 IU; vitamin D_3, 500 IU; vitamin E, 40 IU; vitamin K_3, 2 mg; vitamin B_{12} , .03 mg; riboflavin, 12 mg; niacin, 40 mg; pantothenic acid, 25 mg; choline, 600 mg; biotin, 0.25 mg; folic acid, 1.6 mg; thiamin, 3.0 mg; Ethoxyquin, 5 mg; Fe, 150 mg; Mn, 20 mg; Zn, 120 mg; Cu, 125 mg; I, .2 mg; Se, .3 mg. Supplied by Hoffmann-LaRoche Ltd., 2455 Meadowprine Blvd., Mississauga, ON.

[d] Provided (%): Available phosphorous, 15-18; calcium, 24. Supplied by Continental Lime Ltd. Exshaw, AB.

[e] Provided (%): Calcium, 38. Supplied by Continental Lime Ltd. Exshaw, AB.

[f] Veterinary LS-20 premix,. Provided (g/kg mixture): Lincomycin hydrochloride, 22, and Spectinomycin sulphate, 22 with mineral oil USP, 10, and soybean meal, 946 as carrier. Supplied by the Upjohn Company, Animal Health Division, Orangeville, ON.

Table V-2. Proximate analyses and amino acid composition (%)[a] of Nutrisoy, autoclaved Nutrisoy and the experimental diets

Items	Nutrisoy	Autoclaved Nutrisoy	Nutrisoy diet	Autoclaved Nutrisoy diet
Dry matter	92.6	92.6	91.9	91.7
Crude protein	57.5	57.2	20.3	20.7
Gross energy (Mcal/kg)	4.8	4.8	4.5	4.5
Neutral-detergent fiber	7.3	7.5	8.0	8.0
Acid-detergent fiber	4.0	4.1	6.6	6.6
Ether extract	1.2	.9	6.1	5.9
SBTI (mg/g)	38.4	9.3	13.3	3.3
Amino acids:				
Indispensable				
Arginine	3.80	3.70	1.38	1.39
Histidine	1.36	1.33	.50	.50
Isoleucine	2.46	2.45	.91	.94
Leucine	3.99	3.98	1.49	1.55
Lysine	3.35	3.09	1.21	1.17
Methionine	.93	.96	.77	.77
Phenylalanine	2.66	2.65	1.00	1.04
Threonine	1.91	1.90	.72	.73
Valine	2.44	2.44	.91	.95
Dispensable				
Alanine	2.24	2.24	.85	.86
Aspartic acid	5.17	5.13	1.96	1.97
Cysteine	.86	.95	1.00	.86
Glutamic acid	9.17	9.09	3.49	3.62
Glycine	2.24	2.24	.84	.86
Serine	2.47	2.46	.94	.96
Tyrosine	1.53	1.56	.52	.55

[a] Dry matter basis.

Table V-3. Effect of experimental diet on ileal and fecal digestibilities (%) of nutrients and gross energy in growing pigs

Items	Ileal digestibilities			Fecal digestibilities		
	Nutrisoy	Autoclaved Nutrisoy	SE[a]	Nutrisoy	Autoclaved Nutrisoy	SE[a]
Dry matter	65.2[c]	74.1[b]	1.41	87.7[c]	89.6[b]	.31
Organic matter	67.8[c]	76.3[b]	1.19	89.6[c]	91.2[b]	.36
Crude protein	41.2[c]	77.6[b]	1.48	79.1[c]	90.1[b]	.43
Gross energy	67.1[c]	78.0[b]	1.25	88.3[c]	90.8[b]	.44
Amino acids:						
Indispensable						
Arginine	48.7[c]	90.3[b]	1.29	86.0[c]	95.6[b]	.44
Histidine	48.4[c]	83.2[b]	1.65	86.6[c]	94.7[b]	.49
Isoleucine	44.1[c]	86.5[b]	1.79	76.4[c]	91.2[b]	.77
Leucine	40.6[c]	87.0[b]	1.45	77.3[c]	91.7[b]	.80
Lysine	45.1[c]	80.2[b]	2.01	81.5[c]	90.1[b]	.64
Methionine	70.0[c]	89.4[b]	2.32	80.1[c]	91.8[b]	1.54
Phenylalanine	41.8[c]	88.1[b]	1.26	79.0[c]	92.7[b]	.75
Threonine	41.3[c]	73.7[b]	3.02	75.0[c]	88.8[b]	.65
Valine	41.7[c]	84.0[b]	1.33	75.9[c]	90.8[b]	.71
Dispensable						
Alanine	47.4[c]	81.7[b]	1.66	74.8[c]	89.2[b]	.85
Aspartic acid	44.9[c]	72.6[b]	.99	82.7[c]	92.4[b]	1.01
Cysteine	39.4[c]	67.6[b]	4.43	78.8[c]	86.2[b]	1.74
Glutamic acid	51.1[c]	84.1[b]	.93	87.2[c]	95.3[b]	.43
Glycine	33.9[c]	70.3[b]	3.56	77.9[c]	89.6[b]	.72
Serine	39.8[c]	81.0[b]	1.54	81.9[c]	92.8[b]	.48
Tyrosine	38.5[c]	85.4[b]	1.54	75.9[c]	91.3[b]	.79

[a] Standard error of the mean (n = 10).

[b,c] Means in the same row, within ileal or fecal digestibilities, with different superscripts differ ($P < .01$).

Table V-4. Effect of experimental diet and experimental period on the net disappearance (g/kg DMI) of nutrients and gross energy in the large intestine of growing pigs

Items	Dietary treatment			Experimental period		
	Nutrisoy	Autoclaved Nutrisoy	SE[a]	Period 1	Period 2	SE[a]
Dry matter	225.0[b]	155.0[c]	14.23	176.7	203.3	1423
Organic matter	206.2[b]	141.0[c]	11.33	161.3	185.9	1133
Crude protein	76.9[b]	25.9[c]	3.27	45.3[c]	57.5[b]	3.27
Gross energy (Mcal/kg)	1.0[b]	.6[c]	.54	.7	.8	.06
Indispensable						
Arginine	5.0[b]	.7[c]	.17	2.3[c]	3.4[b]	.17
Histidine	1.8[b]	.6[c]	.09	1.2	1.2	.09
Isoleucine	3.0[b]	.4[c]	.19	1.2[c]	2.3[b]	.19
Leucine	5.5[b]	.7[c]	.21	2.3[c]	3.8[b]	.21
Lysine	4.4[b]	1.2[c]	.22	2.5	3.1	.22
Methionine	3.2[b]	.4[c]	.11	1.4[c]	2.2[b]	.11
Phenylalanine	3.7[b]	.5[c]	.13	1.6[c]	2.6[b]	.13
Threonine	2.4[b]	1.1[c]	.22	1.4[c]	2.1[b]	.22
Valine	3.2[b]	.7[c]	.13	1.4[c]	2.5[b]	.13
Dispensable						
Alanine	2.3[b]	.6[c]	.15	1.0[c]	1.9[b]	.15
Aspartic acid	7.4[b]	4.0[c]	.29	4.7[c]	6.7[b]	.29
Cysteine	3.3[b]	.6[c]	.13	1.4[c]	2.6[b]	.13
Glutamic acid	12.7[b]	4.0[c]	.34	6.8[c]	9.9[b]	.34
Glycine	3.7[b]	1.6[c]	.31	2.3	3.0	.31
Serine	3.9[b]	1.1[c]	.14	2.1[c]	2.9[b]	.14
Tyrosine	2.0[b]	.3[c]	.10	.9[c]	1.4[b]	.10

[a] Standard error of the mean (n = 10).

[b, c] Means in the same row, within treatments or periods, with different superscript letters differ ($P < .01$).

Table V-5. Effects of experimental period on ileal and fecal digestibilities (%) of nutrients and gross energy in growing pigs

Items	Ileal digestibilities			Fecal digestibilities		
	Period 1	Period 2	SE[a]	Period 1	Period 2	SE[a]
Dry matter	69.8	69.5	1.42	87.5[c]	89.8[b]	.31
Organic matter	72.2	71.9	1.20	89.6[c]	91.2[b]	.36
Crude protein	60.1	58.7	1.48	82.3[c]	86.9[b]	.43
Gross energy	72.7	72.4	1.25	88.3[c]	90.7[b]	.44
Amino acids:						
Indispensable						
Arginine	70.9	68.1	1.29	88.3[c]	93.3[b]	.44
Histidine	63.2	68.4	1.65	88.1[c]	93.2[b]	.49
Isoleucine	67.1	63.5	1.79	80.1[c]	87.5[b]	.77
Leucine	65.1	62.4	1.79	80.9[c]	88.2[b]	.80
Lysine	61.9	63.4	2.01	82.7[c]	88.9[b]	.64
Methionine	80.1	79.3	2.32	81.6[c]	90.4[b]	1.54
Phenylalanine	66.4	63.1	1.26	82.4[c]	89.3[b]	.75
Threonine	58.7	56.3	3.02	78.4[c]	85.4[b]	.64
Valine	65.3[c]	60.4[b]	1.33	79.8[c]	87.0[b]	.71
Dispensable						
Alanine	65.8	63.3	1.69	77.9[c]	86.0[b]	.85
Aspartic acid	61.7[b]	55.9[c]	1.00	85.5[c]	89.6[b]	1.01
Cysteine	70.2[b]	65.0[c]	.93	89.3[c]	93.2[b]	.43
Glycine	53.0	51.2	3.56	80.6[c]	86.9[b]	.72
Serine	62.4	58.9	1.54	84.7[c]	90.1[b]	.48
Tyrosine	63.0	60.9	1.54	79.9[c]	87.4[b]	.78

[a] Standard error of the mean (n = 10).

[b, c] Means in the same row, within ileal or fecal digestibilities, with different superscripts differ ($P < .01$).

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CHAPTER VI

EFFECT OF DIETARY PROTEIN CONTENT ON TOTAL VOLUME, PROTEIN CONTENT AND ENZYME ACTIVITIES OF PANCREATIC JUICE COLLECTED FROM GROWING PIGS

A. Introduction

There is a scarcity of information on the effect of dietary CP levels on the total secretion of protein and enzyme activities in exocrine pancreatic secretions. One study (Hee et al., 1988), with pigs fed a corn starch-based diet containing 15% CP from soybean meal or a corn starch-based protein-free diet, reported no differences ($P > .05$) in the total secretion of protein and lipase activity. However, the total activities of trypsin, chymotrypsin and amylase were considerably lower ($P < .05$) in pigs fed the protein-free diet. The results reported by Hee et al. (1988) were not surprising because the pigs were in a protein-deficient condition. In another study, Corring and Saucier (1972) fed four corn starch-based diets containing 0, 10, 30 and 40% CP from fish meal, included at the expense of of corn starch. They reported an increase in protein output and enzyme activities in pancreatic juice as the dietary CP content was increased. However, the results from their study do not allow for comparison of total secretion of protein and enzyme activities in pancreatic juice between the four diets. In their design, the diets were fed in a sequence (i.e. not randomized). As was shown previously (Chapter IV), pig BW per sé affects pancreatic secretions.

The objective of this study was to determine the effect of dietary CP level on the exocrine pancreatic secretions of protein and enzyme activities in growing pigs prepared with the "Pouch" technique (Hee et al., 1985) which allows for long term and total collection of pancreatic juice. Two corn starch-based model diets, containing 12 and 24% CP from soybean meal, were evaluated in this study. The amino acid supply in both diets met or exceeded NRC (1988) requirements. The dietary CP content was chosen at 12 to 24%, as

this range encompasses the CP content in diets for pig of all ages.

B. Experimental Procedures

Animals and diets

Six PIC barrows (Camborough x Canabrid), average BW 20.2 ± .9 kg, were obtained from the University of Alberta Swine Research Unit. The pigs were housed individually in stainless steel metabolism crates (length: 140 cm; height: 85 cm; width: 80 cm) in a barn in which the temperature was maintained at 25 ± 1° C; light was on continuously. The pigs were given *ad libitum* access to a 16% CP grower diet (Sauer et al., 1983). Water was freely available through a low-pressure drinking nipple.

After two days, the pigs were fitted with a pancreatic re-entrant cannula at the duodenum for total collection of pancreatic juice. Procedures for pre-operative, surgery and post-operative care were carried out according to Hee et al. (1985). The pancreatic re-entrant cannula was constructed according to Hee et al. (1985) with modifications described by Ozimek et al. (1986). The pigs were immediately returned to the metabolism crates after surgery and fasted that same day. The next day they were given approximately 100 g of an 18% CP starter diet ((Sauer et al., 1983) twice daily. The daily feed allowance was gradually increased until the pigs consumed the diet at a daily rate of 5% (wt/wt) of their individual BW. The re-entrant cannula and the connecting one-way valve was flushed with saline twice daily during the first three days after surgery to ensure that there was no blockage. The pigs were allowed a 14-d recuperation period, during which the starter diet was fed.

Two corn starch-based soybean meal diets were formulated to contain 12% (LCP) and 24% crude protein (HCP) from soybean meal, respectively (Table VI-1). The soybean meal (47% CP) was solvent-extracted and ground through a 2-mm mesh screen prior to incorporation into the diets. Solkafloc (4.5%) and canola oil (2.5%) were included in the diets. Dextrose (10%) was included to improve the palatability. Vitamins and minerals were included to meet or exceed the NRC (1988) standards. Lysine, threonine and methionine

were also included in the LCP diet to meet the NRC (1988) standards.

The pigs were allotted to the two experimental diets according to a crossover design (Petersen, 1985). Each experimental period lasted 12 d. The pigs were fed twice daily, at 0800 and 2000, equal amounts each meal in the form of mash. The daily allowance was offered at a rate of 5% (wt/wt) of BW for each pig which was measured at the beginning of each experimental period. The average BW were 24.0 ± 1.1 kg and 31.3 ± 2.6 kg at the start of periods 1 and 2, respectively. The average BW at the conclusion of the experiment was 40.2 ± 1.6 kg. The experimental proposal and surgical procedures were reviewed and approved by the Animal Care Committee of the Faculty of Agriculture, Forestry, and Home Economics at the University of Alberta. The animals were cared for in accordance with the guidelines established by CCAC (1980).

Collection of pancreatic secretions

Pancreatic juice was collected continuously for 12 h from 0800 to 2000 h on day 1, 3, 5, 8 and 10 of both experimental periods. The collection, sampling and return of pancreatic juice to the pigs were carried out according to Hee et al. (1985). Samples of pancreatic juice, 10% of the hourly volume, were taken each h during the 12 h collection period and immediately frozen at -20° C.

Chemical and Statistical Analyses

Samples of the diets were taken each time the meal allowances were weighed out and finally pooled for each dietary treatment. Samples of the diets were ground in a Wiley mill through a .8-mm mesh screen before analysis. Analyses for DM, CP and EE were carried out according to AOAC (1984). Analyses for NDF and ADF were carried out according to procedures outlined by Goering and van Soest (1970). The GE content was determined with an AC-300 Leco Automatic Calorimeter (Leco Corporation, St. Joseph, MO). Amino acid contents of the diets were determined according to procedures previously described by Li et al. (1993) and in Chapter III. The chemical and amino acid composition of the experimental diets are presented in Table VI-2.

For the determination of protein content and enzyme activities, the hourly samples of pancreatic juice were thawed at 4° C and pooled, taking 2% of each hourly sample, for each pig and each 12-h collection period. Total nitrogen in pancreatic juice was measured with a Leco FP-428 Nitrogen Analyzer (Leco Corporation, St. Joseph, MO). The protein content in pancreatic juice was determined according to procedures described by Lowry et al. (1951). The standard curve was prepared using bovine serum albumin (Sigma Chemical Company, St. Louis, MO; Code: P-0914) as standard. The pooled pancreatic juice was diluted 83.3-fold (1.2 mL juice was diluted to 100 mL with deionized water) and kept at 4° C. Three mL copper reagent and 1.2 mL diluted pancreatic juice were pipetted into each test tube, mixed thoroughly, and kept at room temperature for 30 to 60 min. Folin color reagent (.3 mL/tube) was added and allowed to stand for another 45 min at room temperature for the color to develop. The optical density was measured at 660 nm against deionized water as reference with a Milton Roy Spectronic 3000 Array Spectrophotometer equipped with an auto-sampling pump (Fisher Scientific, Edmonton, AB, Canada).

Amylase (EC 3.2.1.1) activity in pancreatic juice was determined according to procedures described by Rick and Stegbauer (1974) and the Enzyme Manual (WBC, 1988); lipase (EC 3.1.1.1) activity according to Schmidt et al. (1974). Activities of trypsin and chymotrypsin in pancreatic juice were determined according to Rick (1974a,b) following activation of chymotrypsinogen and trypsinogen to chymotrypsin and trypsin, respectively, by enterokinase (Sigma, enteropeptidase, EC 3.4.21.9; Code: E-0632). The activation procedures were carried out according to Glazer and Steer (1977) with modifications described by Gabert et al. (1995). Firstly, .2 mL undiluted pancreatic juice was added to 1.8 mL of buffer (pH 8.1) containing 100 µg/mL bovine serum albumin (Sigma; Code: A-3059), 50 mM $CaCl_2$ and 50 mM tris-[hydroxymethyl]-aminomethane (Sigma 7-9; Code: T-1378). The enterokinase was dissolved in distilled and deionized water (10 mg/mL) and centrifuged at 15,600 x *g* at 5° C for 15 min to remove cell debris. Activation was initiated by adding .2 mL of the diluted pancreatic juice to .2 mL of supernatant which contains the enterokinase followed by incubation at 5°C for 3 and 120 h for chymotrypsin and trypsin, respectively.

Enzyme activities in pancreatic juice were expressed as units (U) per litre (U x

10^{-3}/L) for specific and U per 24 h (U x 10^{-3}/24 h) for total activity. One U of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 μM of substrate in 1 min at 25° C. Total enzyme activity was calculated as the product of specific activity and total volume of pancreatic juice secreted in 24 h. Previous studies with pigs (Hee et al., 1988; Pöhland et al., 1993) showed no differences ($P > .05$) in secretion volume, protein content and enzyme activities of pancreatic juice collected between day (0800 to 2000) and night (2000 to 0800). Therefore, results obtained from a 12-h collection multiplied by two represent results from a 24-h collection of pancreatic juice. Results from the last two collections (d 10 and d 12 of each experimental period) were used to determine the effect of dietary CP level on the total volume, protein and enzyme activities of pancreatic juice.

Results were subjected to analysis of variance using the General Linear Model procedure of SAS (1990). The statistical model included dietary treatments, experimental periods, animals and interactions between dietary treatments and experimental periods and animals as sources of variation. The means of dietary treatments and experimental periods were compared with the Students' *t*-test (Steel and Torrie, 1980).

C. Results and Discussion

The pigs remained healthy and consumed their meal allowances in less than one h after feeding throughout the experiment. Postmortem examinations, conducted at the conclusion of the experiment, revealed no intestinal adhesions or other abnormalities.

The effect of dietary protein level on the volume of pancreatic juice is presented in Table VI-3. The total volume of pancreatic secretion was slightly higher ($P < .05$) when the LCP diet was fed. The volume of secretions of pancreatic juice in this study was less than those reported by Hee et al. (1988) and Pöhland et al. (1993) in studies with larger pigs, but similar to those reported by Gabert et al. (1995) with pigs in the same BW range that were also fed corn starch-based soybean meal diets.

The concentration of protein in pancreatic juice (g/L) was higher ($P < .05$) when the HCP diet was fed (Table VI-3). There was no effect ($P > .05$) of dietary CP level on the total

secretion (g/24 h) of protein. The daily protein output in pancreatic juice was approximately 10 g, which accounted for only 6.0 and 3.4% of the dietary CP intake of pigs fed the LCP and HCP diets, respectively. From this study, it seems that pancreatic secretion of protein is not affected by the dietary CP level. It should be pointed out that the amino acid supply in the LCP diet, which was supplemented with lysine, methionine and threonine, met the NRC (1988) requirements. This may explain the absence of response of pancreatic protein secretion to differences in dietary CP contents. As was suggested by Imbeah et al. (1988), only very large differences in dietary CP content will perhaps elicit differences in pancreatic protein output.

The specific activity of chymotrypsin was higher ($P < .01$) when the HCP diet was fed (Table VI-3), however, its total activity was not affected ($P > .05$) by dietary CP content. In addition, there was no effect ($P > .05$) of dietary CP level on both specific and total activities of trypsin. The effect of dietary CP content on the secretion of the proteolytic enzymes, using the same protein source, has not been investigated yet. Studies by Pöhland et al. (1993), with pigs fed diets with CP contents ranging from 11.2 to 18.6% but from different protein sources, also showed no effect ($P > .05$) on the total activities of chymotrypsin and trypsin.

The specific activity of amylase was higher ($P < .01$) in pancreatic juice from pigs fed the HCP diet (Table VI-3). However, there was no effect ($P > .05$) of dietary CP content (or inversely of starch content) on the total activity of this enzyme. The contents of corn starch in the LCP and HCP diets were 52.0 and 27.2%, respectively (Table VI-1). These results do not agree with those reported by Corring (1975) who observed a direct relationship between dietary starch content and amylase secretion.

In addition, there was also no effect ($P > .05$) of dietary CP content on the specific and total activities of lipase (Table VI-3). These results were anticipated because the contents of EE were similar in both diets (Table VI-2). Theoretically, it is expected that the enzyme activities in pancreatic juice vary depending on the concentration of their respective substrates provided in the diet. However, it seems that lipase activity remains relatively constant unless the dietary substrate concentration of EE changes substantially. As was

reported by Corring (1977), an increase of peanut oil in the diet from 3 to 21% resulted in an increase of 230% in lipase activity.

The present study showed that there was no effect ($P > .05$) of dietary CP level (12 vs 24%) on the total secretion of protein and enzyme activities in pancreatic juice. Hee et al. (1988) fed pigs a corn starch-based diet that contained 15% CP from soybean meal or a corn starch-based protein-free diet. There were no differences ($P > .05$) in the total secretion of protein in pancreatic juice and lipase activity. However, the total activities of trypsin, chymotrypsin and amylase in pancreatic juice were considerably lower ($P < .05$) in pigs fed the protein-free diet. Furthermore, Corring and Saucier (1972) fed pigs four corn starch-based diets containing 0, 10, 30 and 40% CP from fish meal, included in the diets at the expense of corn starch. They reported an increase in protein output in pancreatic juice as the dietary CP content was increased. However, the results from their studies do not allow for comparison of total secretion of protein and enzyme activities in pancreatic juice between the four diets, because in their study, the diets were fed in sequence (i.e. at different BW of the pigs). As was shown previously (Chapter IV) and also in this study, the BW of pigs has a considerable effect on pancreatic protein and enzyme activities.

The effect of experimental period on the total volume, protein content and enzyme activities of pancreatic juice are presented in Table VI-4. The average BW of the pigs were 27.6 and 35.8 kg for experimental periods 1 and 2, respectively. The total volume of secretion of pancreatic juice and protein were higher ($P < .01$) in period 2 than 1. With respect to the enzymes, the specific activities of all enzymes, with the exception of trypsin, were lower ($P < .01$) in period 2, most likely due to the dilution effect of the higher secretion volume of pancreatic juice during the second experimental period. On the other hand, the total activities of these enzymes were higher in period 2. The differences were significant ($P < .01$) for chymotrypsin and lipase. These results are in general agreement with those presented in Chapter IV in which the volume of pancreatic secretion and total protein secretion were higher ($P < .05$) during the second experimental period. Furthermore, even though the differences were not significant, the total activities of amylase and chymotrypsin were higher in period 2 than 1. These results confirm the findings of Weström et al. (1988);

as BW increases secretion rate increases. Gabert et al. (1995) also reported an increase in the secretion volume, protein secretion and activities of some of the pancreatic enzymes between experimental periods in the same experiment.

Protein in pancreatic juice is one of the endogenous protein sources secreted into the digestive tract. As this study shows, the secretion of total pancreatic protein is not affected by dietary CP content, over the range from 12 to 24%. A recent study by Fan et al. (1995), with growing pigs using the regression method, showed that the level of endogenous protein (amino acids) collected from the distal ileum was constant at different dietary protein levels. Results from this study support the findings by Fan et al. (1995). However, it should be pointed out that the effect of dietary CP content was only measured for one source of endogenous protein, not for others which include sloughed epithelial cells, mucus and intestinal secretions.

D. Implications

Dietary CP content, 12 vs 24%, did not affect the total secretion of protein and total enzyme activities in pancreatic juice. Furthermore, the volume of pancreatic secretion, total protein content and enzyme secretions in pancreatic juice are dependent on BW of pigs.

Table VI-1. Formulation (%) of the low and high crude protein experimental diets[a]

Ingredients	Diets	
	Low protein[b]	High protein[b]
Soybean meal	25.5	51.0
Corn starch	52.0	27.2
Dextrose	10.0	10.0
Solkafloc	4.5	4.5
Canola oil	2.6	2.5
Biophos[c]	2.2	2.2
Calcium Carbonate[d]	.9	.9
Vitamin-mineral premix[e]	1.0	1.0
Iodized salt[f]	.3	.3
Chromic oxide	.3	.3
Antibiotics (LS 20)[g]	.1	.1
L-lysine (78%)	.3	-
L-threonine (98%)	.2	-
DL-methionine (98%)	.1	-

[a] As-fed basis.

[b] Low crude protein diet (LCP, 12% CP); High crude protein diet (HCP, 24% CP).

[c] Provided (%): available phosphorous, 15-18; calcium, 24. Supplied by Continental Lime Ltd., Exshaw, AB.

[d] Provided 38% calcium. Supplied by Continental Lime Ltd. Exshaw, AB.

[e] Provided the following (per kg diet): vitamin A, 7,500 IU; vitamin D_3, 500 IU; vitamin E, 50 mg; vitamin K_3, 2.0 mg; vitamin B_{12} , .03 mg; riboflavin, 12 mg; niacin, 40 mg; pantothenic acid, 25 mg; choline, 600 mg; biotin, .25 mg; folic acid, 1.6 mg; thiamine, 3.0 mg; Ethoxyquin, 5.0 mg; pyridoxine, 2.25 mg. Cu, 125 mg; Fe, 150 mg; I, .21 mg; Se, .3 mg; Mn, 20 mg; Zn, 150 mg. Supplied by Hoffmann-LaRoche Ltd. 2455 Meadowprine Blvd. Mississauga, ON.

[f] Provided (%): NaCL, 99.0; Iodine, .15. Supplied by Sifto Canada Inc., Mississauga, ON.

[g] Veterinary LS-20 premix. Provided (g/kg mixture): Lincomycin hydrochloride, 22, Spectinomycin sulphate, 22, with mineral oil USP, 10 and soybean meal, 946 as carrier. Supplied by the Upjohn Company. Animal Health Division, Orangeville, ON.

Table VI-2. Proximate analyses and amino acid composition (%) of the experimental diets[a]

Items	Diets	
	Low protein[b]	High protein[b]
Dry matter	90.9	91.3
Crude protein	13.4	26.0
Gross energy (Mcal/kg)	3.9	4.1
Ether extract	3.9	3.9
Neutral-detergent fiber	6.3	8.5
Acid-detergent fiber	5.0	6.1
Amino acids:		
Indispensable		
Arginine	.96	1.94
Histidine	.34	.70
Isoleucine	.63	1.23
Leucine	1.03	1.98
Lysine	1.03	1.72
Phenylalanine	.67	1.34
Threonine	.70	.98
Valine	.63	1.24
Dispensable		
Alanine	.57	1.11
Aspartic acid	1.41	2.74
Glutamic acid	2.34	4.57
Glycine	.59	1.16
Serine	.63	1.16
Tyrosine	.33	.73

[a] Dry matter basis.
[b] Refer to Table VI-1.

Table VI-3. Total secretion volume, protein contents and enzyme activities of pancreatic juice collected from growing pigs fed low and high protein diets.

Items		Diets		
		Low protein[a]	High protein[a]	SE[b]
Volume	(mL/24h)	2410.4[d]	2229.0[e]	33.97
Protein[c]	(g/L)	4.2[e]	4.8[d]	.13
	(g/24h)	10.0	10.1	.53
Enzyme activities:				
Chymotrypsin	($Ux10^{-3}$/L)	44.8[E]	55.9[D]	.46
	($Ux10^{-3}$/24h)	107.2	119.7	4.73
Trypsin	($Ux10^{-3}$/L)	43.4	53.5	8.49
	($Ux10^{-3}$/24h)	94.8	103.0	12.34
Amylase	($Ux10^{-3}$/L)	553.0[E]	630.6[D]	12.72
	($Ux10^{-3}$/24h)	1276.0	1265.3	129.90
Lipase	($Ux10^{-3}$/L)	44.2	44.4	1.35
	($Ux10^{-3}$/24h)	105.2	93.8	3.04

[a] Refer to Table VI-1.
[b] Standard error of the mean (n = 12).
[c] Determined according to Lowry et al. (1951).
[d, e] Means in the same row with different superscript letters differ ($P < .05$).
[D, E] Means in the same row with different superscript letters differ ($P < .01$).

Table VI-4. Effect of experimental period on secretion volume, protein contents and enzyme activities of pancreatic juice collected from growing pigs

Items		Period 1	Period 2	SE[a]
Volume	(mL/24h)	1761.8[C]	2877.7[B]	33.97
Protein	(g/L)	4.6	4.4	.13
	(g/24h)	7.6[C]	12.4[B]	.53
Enzymes:				
Chymotrypsin	($Ux10^{-3}$/L)	52.2[B]	48.5[C]	.46
	($Ux10^{-3}$/24h)	88.9[C]	138.1[B]	4.73
Trypsin	($Ux10^{-3}$/L)	60.9	36.0	8.49
	($Ux10^{-3}$/24h)	97.4	100.3	12.34
Amylase	($Ux10^{-3}$/L)	665.7[B]	517.9[C]	12.72
	($Ux10^{-3}$/24h)	1091.0	1450.3	129.93
Lipase	($Ux10^{-3}$/L)	48.6[B]	39.9[C]	1.35
	($Ux10^{-3}$/24h)	84.2[C]	114.7[B]	3.04

[a] Standard error of the mean (n = 12).

[B, C] Means in the same row with different superscript letters differ ($P < .01$).

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CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

An interest in exogenous enzyme supplementation and exocrine pancreatic enzyme secretions and their impacts on nutrient digestibility in pigs initiated the studies included in this thesis.

As was reviewed in several reports (Chesson, 1987, 1993; Dierick, 1989), supplementation of exogenous enzymes with ß-glucanase activity to barley-based diets for broilers has become common practice, because positive responses in terms of FE and ADG have been established. However, studies with pigs have shown inconsistent results. As was reviewed in Chapter I, some studies showed improvements in nutrient and GE digestibilities in growing pigs (Graham et al., 1988; Thacker et al., 1988), whereas others showed no improvement in both young (Bedford et al., 1992; Thacker et al., 1992b) and growing pigs (Graham et al., 1986; Thacker et al., 1992a). Caution should be exercised when comparing results from different studies, because the sources of enzyme products (Table I-1) and barley used in the aforementioned studies may contribute to differences in response. If it is true that bacterial degradation of ß-glucans in older pigs compromises the effect of ß-glucanase supplementation, then there should be a positive effect of enzyme supplementation on nutrient and GE digestibilities in very young pigs in which the microbial population in the digestive tract is not fully developed yet.

In addition to exogenous enzyme supplementation, exocrine pancreatic enzyme secretion is a crucial determinant in digestion. To investigate the digestive enzyme secretion in pigs, three studies were carried out with growing pigs fed model diets containing low and high levels of SBTI from Nutrisoy and diets containing low and high levels of CP from soybean meal.

The detrimental effects of feeding raw soybean, which has a high content of SBTI, on pancreatic function and performance of rats and chicks have been well documented in the

literature (e.g., Rackis et al., 1979; Liener and Kakade, 1980). Rats and chicks fed raw soybean or SBTI respond by hypersecretion of pancreatic enzymes and/or hypertrophy of the pancreas (Garlich and Nesheim, 1966; Rackis, 1972). With regard to the pancreatic secretions in pigs, as affected by feeding raw soy products or SBTI, most studies reported in the literature focused either on performance (e.g., Combs et al., 1967; Yen et al., 1974) or nutrient digestibility (Combs et al., 1967; Vandergrift et al., 1983). Some studies have dealt with enzyme activities in pigs fed raw soybean (e.g., Yen et al., 1977), however, the results from these studies were based on enzyme activities in intestinal contents or pancreatic tissue rather than pancreatic juice. As was previously pointed out by Hee et al. (1985, 1988a,b), it is essential to consider total rather than specific activity which is usually expressed as U/g tissue or U/g digesta. Total rather than specific enzyme activity determines the extent of digestion.

There is a scarcity of information on the effect of SBTI on pancreatic secretions, using total collection of pancreatic juice, in pigs. Therefore, studies were carried out to determine the effect of SBTI on pancreatic secretions in pigs prepared with the "Pouch" technique (Hee et al., 1985). The use of this technique allows for long term and total collection of pancreatic juice. In addition, studies were carried out to determine the effect of SBTI on both ileal and fecal digestibilities of nutrients and GE in growing pigs.

A. ß-glucanase Supplementation to Diets for Young Pigs

Two experiments were carried out to study the effect of ß-glucanase supplementation to hulless barley-, wheat-, corn- and rye-based soybean meal diets on nutrient and GE digestibilities with young pigs (Chapters II and III). Results showed that ß-glucanase (a mixture of enzymes with endo- and exo-ß-glucanase and ß-glucosidase activities; 1,000 fibrinolytic activity units/g) supplementation to the B+SBM diet improved ($P < .05$) the apparent fecal digestibilities of DM, CP and GE in young pigs (BW 6.2-11.2 kg). There was no response ($P > .05$) of ß-glucanase supplementation to the W+SBM, C+SBM and R+SBM

diets, possibly due to the low ß-glucan contents in these diets. ß-glucanase supplementation also improved ($P < .05$ or $P < .01$) the ileal digestibilities of DM, OM, CP, GE, ß-glucans, and the majority of amino acids in the B+SBM but not in the W+SBM diet in young pigs (BW 7.3 - 11.4 kg).

The improvements in nutrient digestibility may be attributed to increased degradation of cell wall components in hulless barley, which resulted in better access of digestive enzymes to their respective substrates and thus more complete digestion of starch and protein in the small intestine (Hesselman and Åman, 1986). The mechanisms by which ß-glucans interfere with digestion and absorption are closely related to their physicochemical properties. ß-glucans differ from cellulose in that they are mixed-linked; approximately 30% of the linkages between glucose units are in the form of ß (1-3) and 70% in the form of ß (1-4) (Fleming and Kawakami, 1977). This branched structure prevents compact folding of the molecules and increases the water-holding capacity which results in its characteristic viscosity and gelling properties. The viscosity and gelling properties tend to hinder intestinal motility (Holt et al., 1979) thereby decreasing the mixing of digesta, digestive enzymes and other necessary components required for digestion and absorption (Vahouny and Cassidy, 1985). These properties may also delay or decrease digestion and absorption of nutrients by increasing the unstirred fluid layer, creating a physical barrier at the absorption surface on the microvilli (Johnson and Gee, 1981).

ß-glucanase supplementation to the B+SBM diet reduced ($P < .05$) the net disappearance of DM, OM, GE and ß-glucans in the large intestine. Although the differences were not significant ($P > .05$), the net disappearance of CP and amino acids showed a similar trend. The reduction in net disappearance in the large intestine indicated that ß-glucanase supplementation resulted in an increase in digestion and absorption of nutrients and GE in the small intestine. The shift of disappearance of GE from the large to the small intestine, in this case resulting from ß-glucanase supplementation, will result in an improvement in the efficiency of energy utilization, as was shown by Just et al. (1983) in studies with pigs fed diets differing in fiber content. In particular, this modification in energy utilization may be

beneficial to young pigs fed diets high in non-starch polysaccharides, for which energy intake may be a limiting factor.

To conclude from the results of the previous studies and those reported in the literature, it seems that supplementation of β-glucanase to barley-based diets for pigs may be beneficial but only for very young pigs. As was demonstrated in studies with growing pigs (Graham et al., 1986), extensive degradation of ß-glucans by the microflora in the digestive tract in growing pigs makes enzyme supplementation meaningless. Further studies are warranted to determine whether the improvements in energy and amino acid digestibilities resulting from β-glucanase supplementation are large enough to be translated into improvements in performance. In addition, studies are warranted to determine the β-glucan degrading activity of the microflora in the digestive tract of the pig and at which BW this activity becomes well established.

B. Exocrine Pancreatic Enzyme Secretions and Their Impact on Nutrient Digestibility in Growing pigs

The effect of feeding diets containing relatively high and low levels of SBTI to growing pigs were investigated with pigs of 33.5 to 41.8 kg BW (Chapter IV). Incorporation of Nutrisoy compared with autoclaved Nutrisoy into corn starch-based diets increased ($P <$ .01) the total volume of pancreatic secretion and decreased ($P <$ .01) the concentrations (U x 10^{-3}/L) of pancreatic amylase, chymotrypsin and trypsin but not ($P >$ .05) of lipase. However, there was no effect on the total activities (U x 10^{-3}/ 24h) of these enzymes. These results are in agreement with those reported by Zebrowska et al. (1985) who observed no effect ($P >$ .01) on the total activities of trypsin, chymotrypsin and amylase when heat-treated compared to raw soybean meal was included in a corn starch-based diet. These results show that the negative feedback regulation mechanism to increase pancreatic secretions of trypsin and chymotrypsin subsequent to feeding SBTI or raw soybean, which is present in chicks and rats, is absent in growing pigs.

Green and Lyman (1972) hypothesized that dietary trypsin inhibitors evoke increased pancreatic enzyme secretions in rats and chicks through a negative feedback regulation mechanism. According to this hypothesis, removal or inactivation of trypsin and chymotrypsin from the duodenum will stimulate enzyme secretion by increasing the level of a circulating stimulant known as cholecystokinin. Trypsin inhibitors induce the feedback regulation indirectly by forming a high affinity inhibitor-enzyme complex thereby inactivating the enzyme. Results of the present study demonstrated that this negative feedback regulation mechanism only occurred in terms of volume but not in total secretion of enzymes. Other studies by Yen et al. (1977) with 12 kg gilts reported an absence of enlargement of the pancreas and reduced ($P < .05$) trypsin and chymotrypsin activities, measured in pancreatic tissue and intestinal contents, following the feeding of raw soybean and SBTI. It is speculated that the poor performance of pigs fed raw soy products or SBTI is due to the inactivation of digestive enzymes which leads to a decrease in enzyme hydrolysis of dietary substrates.

The lack of pancreatic enzyme hypersecretion in pigs, in contrast to rats and chicks, was postulated to be related to the ratio of weight of the pancreas to BW of the animal (Liener and Kakade, 1980). Therefore, animals with a larger pancreas, as a percentage of BW, have a higher requirement for the sulphur-containing amino acids needed for the synthesis of pancreatic enzymes than do animals with a smaller pancreas such as pigs and calves. For this reason, animal with a lower pancreas to BW ratio, pigs for instance, are less responsive to the stimulation by trypsin inhibitors. Similar findings were also reported by Batterham et al. (1993) who observed threshold levels of 4.7 and 4.5 mg/g trypsin and chymotrypsin inhibitors in diets containing chickpea meals in 20 kg pigs.

The concentration (mM/L) of total amino acids and the majority of protein-bound and free amino acids in pancreatic juice are lower ($P < .05$) in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. This corresponds to the protein and enzyme contents. Of the indispensable amino acids, the most abundant are leucine, valine, methionine and lysine; of the dispensable amino acids, aspartic acid, glycine, glutamic acid, serine, cysteine and alanine. These observations are in agreement with those reported by Gabert et al. (1995).

In the protein-bound amino acid pool, the most abundant amino acids are aspartic acid, glycine, serine, glutamic acid, cysteine, valine, alanine, threonine, leucine and lysine. It is interesting to note, with the exception of alanine, that all of these amino acids were lower ($P < .05$) in pancreatic juice collected from pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. These amino acids are present in relatively large amounts in digestive enzymes. For example, chymotrypsin contains high amounts of alanine, aspartic acid, glycine and alanine (Charles et al., 1967); trypsin contains high levels of alanine, aspartic acid, glycine, glutamic acid, serine and alanine (Charles et al., 1963). Amylase and lipase also contain high levels of the aforementioned amino acids (Cozzone et al., 1970; Winkler et al., 1990).

It is interesting to note that while total nitrogen secretions in pancreatic juice were similar between both dietary treatments, the true protein contents in pancreatic juice were higher ($P < .05$) in pigs fed the autoclaved Nutrisoy diet. This finding shows that there is more non-protein nitrogen in pancreatic juice collected from pigs fed the Nutrisoy diet.

A further study was carried out with 53.3 to 71.8 kg pigs fed the same Nutrisoy and autoclaved Nutrisoy diets to determine the ileal as well as fecal digestibilities of GE, CP and amino acids (Chapter V). The ileal as well as fecal digestibilities of GE, CP and amino acids were considerably lower ($P < .01$) in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. Among the parameters determined, digestibilities of CP and amino acids showed the largest differences between dietary treatments, with an average of 36.4 percentage units for CP and a range from 19.4 to 46.4 percentage units for the indispensable amino acids. Similar ileal digestibility values of amino acids were reported by Vandergrift et al. (1983). The reduced ileal digestibilities of these nutrients and GE resulted in a lower ADG and FE. The ADG of pigs fed the Nutrisoy and autoclaved Nutrisoy diets were 523 and 795 g, and the feed/gain ratio were 4.98 and 2.77, respectively. These results confirmed our previous observations (Chapter IV) in that diets high in SBTI markedly inactivated the pancreatic enzyme activities, which directly decreased nutrient and GE digestibilities, and consequently resulted in poor performance. Depressed performance, reduced N-retention and nutrient

digestibilities were also reported in young (Yen et al., 1974; Cook et al., 1988), growing (Combs et al., 1967) and growing-finishing pigs (Jimenez et al., 1963; Young, 1967), when raw soybean diets were fed. Feeding the Nutrisoy diet also decreased ($P < .01$) the fecal digestibilities of the parameters measured. However, the magnitude was relatively small, with an average of 11.0 percentage units for CP and a range of 8.1 to 14.9 percentage units for the indispensable amino acids.

Compared with the autoclaved Nutrisoy diet, the Nutrisoy diet increased ($P < .01$) the net disappearance of DM, OM, CP, GE and all amino acids in the large intestine. The net disappearance of CP in the large intestine was 76.9 and 25.9 g/kg DMI for the Nutrisoy and autoclaved Nutrisoy diets, respectively. The net disappearance of amino acids followed the same pattern but the magnitude was greater than previously reported by Li et al. (1994).

The high disappearance values of CP, GE and amino acids in the large intestine of pigs fed the Nutrisoy diet further confirm that there is inhibition of proteolysis in the small intestine. Since the net disappearance of GE and nutrients in the large intestine is a direct measurement of the quantity of these nutrients and GE that were undigested in the small intestine and then carried over to the large intestine where they are subjected to microbial fermentation, the higher the disappearance values, the less nutrients will be available to the host animal. Nitsan and Liener (1976) reported that the absorption of amino acid was delayed to a more distal site in the digestive tract of rats fed raw soybean. In the heat-treated soy flour group the concentration of all amino acids in digesta began to decline in the jejunum, whereas in the raw soy flour group the decline began only when protein reached the ileum. Due to this delay, the absorptive capacity of the ileum was probably limited and could not compensate for the absorptive capacity of the jejunum and ileum combined.

The losses of CP, GE and amino acids from the large intestine, due to feeding of the Nutrisoy diet, consisted of the major part of exogenous nutrient losses. Studies with miniature pigs (Barth et al., 1993), using homoarginine as marker, demonstrated that the ingestion of protease inhibitor increased ($P < .05$) the ileal protein output; this increase resulted mainly from endogenous protein. This finding is in contradiction with our results (Chapters IV and

V). The total secretion of pancreatic protein, expressed as g/24h per kg BW, were .80 and .62 for pigs fed the Nutrisoy and autoclaved Nutrisoy diet, respectively. Total protein recovered in ileal digesta were 3.92 and 1.49 for pigs fed the Nutrisoy and autoclaved Nutrisoy diet, respectively. Even if all pancreatic protein secreted in pigs fed the Nutrisoy diet would form complexes with SBTI, which is unlikely, only 20.3% of protein in ileal digesta would be of pancreatic origin.

Further studies were carried out, using homoarginine as internal marker, to determine the ileal recovery of endogenous versus exogenous protein in growing pigs fed the same Nutrisoy and autoclaved Nutrisoy diets. Results of this study were not included in this thesis.

Finally, a study was carried out to determine the effect of dietary CP content on pancreatic secretions in pigs of 24.0 to 40.2 kg BW (Chapter IV). The pigs were fed two corn starch-based soybean meal diets containing 12% CP (LCP) and 24% CP (HCP), respectively. Results of this study demonstrated that the secretion volume of pancreatic juice was slightly higher in pigs fed the LCP than HCP diet. Although protein concentration (g/L) of pancreatic juice was higher ($P < .05$) in pigs fed the HCP than LCP diet, there was no effect ($P > .05$) of dietary CP level on the total protein secretion (g/24 h) in pancreatic juice. The total daily protein outputs in pancreatic juice were 10.0 and 10.1 g for the LCP and HCP diets, respectively. This indicates that total protein secretion in pancreatic juice remains relatively constant regardless of dietary protein content, when the amino acid requirements are met.

The specific activities (U x 10^{-3}/L) of amylase and chymotrypsin increased ($P < .01$) with an increase in dietary CP content. There were no differences ($P > .05$) in the total activities of these enzymes. This finding may be explained by the fact that protein provided by both the LCP and HCP diets met the maintenance requirement for protein synthesis and normal levels of secretions of enzymes in pancreatic juice. It is conventionally stated that protein output in pancreatic secretion increases with dietary CP content. This may only be true when the protein (amino acid) supply is very much below its requirement.

Pancreatic secretion volume and total protein content were higher ($P < .01$) during experimental period 2 than 1, so were the specific and total activities of most pancreatic

enzymes, except for trypsin. No significant differences, both for specific and total activities of this enzyme, were detected between dietary treatments. The reason remains unknown.

C. General Conclusions

To summarize, the following conclusions can be drawn:

1. ß-glucanase supplementation to a hulless barley-based soybean meal diet increased the digestibilities of ß-glucans, GE, CP and the majority of the amino acids. There was a shift of net disappearance of nutrients and GE from the large to the small intestine in the enzyme supplemented diets.
2. The Nutrisoy diet, which contains a relatively higher level of SBTI than the autoclaved Nutrisoy diet, increased the secretion volume of pancreatic juice, but decreased the concentrations (g/L) of nitrogen and protein and the specific activities (U x 10^{-3}/L) of proteolytic enzymes and amylase. Amino acid contents in pancreatic juice were lower in pigs fed the Nutrisoy compared with the autoclaved Nutrisoy diet. However, with the exception of protein, the total contents of nitrogen (g/24 h) and total activities (U x 10^{-3}/24 h) of enzymes were not affected by dietary treatment. No trypsin inhibitor-induced negative feedback regulation of pancreatic enzyme secretion was observed.
3. Compared with the autoclaved Nutrisoy, feeding the Nutrisoy diet decreased both ileal and fecal digestibilities of DM, OM, GE, CP and amino acids. The net disappearance of GE and nutrients in the large intestine was higher in pigs fed the Nutrisoy diet. These high net disappearance values represent a large proportion of energy and protein (and/or amino acids) losses from the animal, in this case, due to the inhibition of proteolysis and absorption in the small intestine. This modification in the pattern of metabolism directly causes a depression in ADG and FE.
4. Dietary protein content (12 vs 24%) did not affect the total secretion of protein and total enzyme activities in pancreatic juice, although the protein concentration (g/L)

and specific activities (U x 10^{-3}/L) of chymotrypsin and amylase were higher in pigs fed the HCP diet. Secretion volume was slightly lower in pigs fed the HCP than LCP diet. Secretion volume, total output of protein and total activities of enzymes in pancreatic juice increased substantially with BW of the animal.

D. Originality and Major Contributions to Knowledge

Some of the findings in this thesis represented an expansion of the frontier of knowledge in the related areas. The following points are claimed to be original contributions to knowledge.

1. ß-glucanase supplementation to a hulless barley-based soybean meal diet fed to young pigs shifted the net disappearance of GE and CP (amino acids) from the large to the small intestine, which will improve the efficiency of energy utilization by young pigs fed diets high in ß-glucan content.
2. Using total collection, pancreatic secretions (volume, nitrogen, protein, amino acids and enzyme activities) were studied in detail in growing pigs fed diets low and high in CP from soybean meal, and diets low and high in SBTI from Nutrisoy.
3. Studies with growing pigs fed the Nutrisoy model diets showed that the trypsin inhibitor-induced negative feedback regulation mechanism in pancreatic enzyme secretion, which occurs in chicks and rats, is absent in growing pigs.
4. In contrast to ß-glucanase supplementation, feeding diets high in SBTI to growing pigs increased the net disappearance of GE, CP and amino acids in the large intestine. This illustrated that the detrimental effect of raw soybean products (or SBTI) on nutrients and GE digestibility is mainly due to losses of exogenous protein resulting from inactivation of proteolytic enzyme activities.

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APPENDIX I

SPECIFICATIONS OF ß-GLUCANASE USED IN THE ENZYME SUPPLEMENTATION STUDIES

A. Name

Generic Name: Cellulase

Commercial Name: Cellulase Kyowa (Kyowa Hakko Kogyo Co., Ltd., Japan)

B. Enzyme Activities

Cellulase Kyowa was prepared from Cellulase Concentrate by dilution with an appropriate vehicle. This enzyme mixture has a high cellulase (Endo-ß-glucanase, Exo-ß-glucanase, ß-glucosidase) activity and also some protease, pectinase, amylase and xylanase activities. Enzymatic activity tests showed that the fibrinolytic activities were not less than 9,000 units/g for Cellulase Concentrate and 1,000 units/g for Cellulase Kyowa. One thousand fibrinolytic activity units are equivalent to the amount of enzyme that disintegrates two pieces of 1 cm^2 filter paper in 1 min at 37° C.

C. Manufacturing Process

Cultivate cellulase producing strain of *T. Reesei.* At the end of cultivation, filter or extract with water and then filter the culture broth to remove the fungal body. Concentrate and dry the filtrate or dry the precipitate condensed by salting-out.

D. Biological and Physicochemical Properties

1. Description:

Cellulase Concentrate is a pale yellow powder having a slightly peculiar odour. The pH of an aqueous solution or water suspension (100-fold dilution) of Cellulase Concentrate

ranges from 4.0 to 7.5. Cellulase Concentrate has a maximum enzymatic activity at pH ranges from 4.0 to 5.0. Loss after drying (1 g, 105° C, 3 h) is not over 10.0%; residues after ashing not over 25.0%.

2. Purity:

Cellulase Concentrate was analyzed for the following:

Heavy metals:	Not over 50 ppm.
Arsenic:	Not over 2 ppm.
Antibacterial activity:	No.

3. Stability:

Cellulase Concentrate and Cellulase Kyowa were kept under the following conditions, 40 °C and 75% relative humidity for three months and their stabilities investigated. The fibrinolytic activity of Cellulase Concentrate did not change; that of Cellulase Kyowa decreased by 10%. Another test showed that the fibrinolytic activity of Cellulase Kyowa decreased by about 10% after oven-drying at 120° C for 60 min.

E. Fibrinolytic Activity Assay

Fibrinolytic activity of cellulase, expressed as fibrinolytic activity units, was measured by reaction with filter paper. One thousand units are equivalent to the amount of enzyme that disintegrates two pieces of 1 cm^2 filter paper at 37° C in 1 min.

Fibrinolytic activity was measured in accordance with the General Test Method of Feed Additives, Testing Method for Fibrinolytic Activity (issued on March 20, 1990 by the University of Agriculture, Forestry and Fisheries, Japan, No. 7).

1. Specifications of filter paper (substrate):

Special grade filter paper made for enzyme activity assay with the following specifications was used as substrate:

Thickness	.29-0.31 mm
Weight	12.5-13.5 mg/cm^2
α-fiber	> 98.5%

Ash	< .05%
Filtering capacity	50-90 seconds/100 mL water
Strength	1.2-1.8 kg/cm^2
Water absorbency	8-9 cm/10 min.
Air permeability	30-40 seconds/cm^2/100 mL.

Select the paper by holding a piece of filter paper to a light, choose a section where the thickness is even and cut into 1 x 1 cm squares.

2. Buffer:

Sodium acetate buffer (1.0 M) was adjusted to pH 4.0-5.0 with 5 M NaOH or acid.

3. Enzymatic hydrolysis:

Prepare an enzyme solution by dissolving an appropriate amount of Cellulase Kyowa in sodium acetate buffer to make a concentration equivalent to 2.8-4.0 fibrinolytic activity units/mL. If the sample does not dissolve completely, leave the solution for one hour with occasional agitation. Then centrifuge the suspension at 2,500 x *g* for 10 min and accurately transfer 5.0 mL of the resultant supernatant into each of five test tubes. The test tubes were kept in a shaking water bath at 37 ± .5° C for 5 min before adding two pieces of 1 cm^2 filter paper. The test tubes are constantly shaken (65 cycles/min, pitch = 60 mm) until all filter paper is disintegrated. Record the time required to completely disintegrate the filter paper into fine fibers.

4. Calculation:

Fibrinolytic activity units/g enzyme sample = 1,000/(T x W)

Where T: Average time (min, exclude the longest and shortest measurements) required for complete disintegration of all substrate filter paper.

W: Weight of enzyme (g) in 5.0 mL supernatant of enzyme solution.

F. Literature Cited

Handbook "Cellulase Kyowa", Kyowa Hakko Kogyo Co., Ltd., Japan.

APPENDIX II

DETERMINATION OF ß-GLUCANS

A. Introduction

The quantitative determination of ß-glucans in cereal grains, particularly barley, is based on the principle that ß-glucans are depolymerized by ß-glucanase followed by release of reducing sugars from ß-glucans; the reducing sugars are quantified using a glucose oxidase system (Anderson et al., 1978), a glucose oxidase-peroxidase kit (Bamforth et al., 1979) or a hexokinase and glucose 6-phosphate dehydrogenase (G-6-P DH) system (Martin and Bamforth, 1981).

Most commercially available enzymes are mixtures which contain not only ß-glucanase but also other enzyme activities. Therefore, commercial products must be pre-treated or purified to eliminate possible contamination of α-amylase and amyloglucosidase activities so that interference by glucose derived from carbohydrates, which are not ß-glucans, in the sample can be prevented. Depending on the physicochemical properties of the respective enzymes, the conventional approaches to purify an enzyme mixture are usually accomplished by means of alteration of pH or temperature. For example, ß-glucanase in commercial cellulase prepared from *Penicillium Funiculosum* is more stable to heat than the equivalent enzyme mixture from *Trichoderma reesei*. Consequently the heat-labile amyloglucosidase in this enzyme preparation can be destroyed after 30 min of heating at 70° C in acetate buffer at pH 4, whereas the ß-glucanase activity remains unchanged even after heating for 60 min under these conditions (Bamforth, 1983).

Furthermore, cereal grains also contain endogenous α-amylase and glucosidase which have a high activity particularly during the germination period. These endogenous enzymes must be inactivated. The steps of sample pre-treatment or sample clean-up are carried out by extracting the sample with 80% ethanol combined with gentle boiling in a

water bath. The boiled sample is then centrifuged and the pellet washed at least three times with 80% ethanol. Extraction with 80% ethanol at boiling temperature has two functions. Firstly, the heat treatment is designed to inactivate the endogenous enzymes in the sample that may interfere with analysis. Secondly, ethanol reduces the soluble oligosaccharides and free sugars to a minimum level (Henry, 1984).

After the ß-glucanase is purified and the samples cleaned up, the samples are hydrolyzed with the purified ß-glucanase under desired pH and temperature conditions. The amount of reducing sugars liberated by ß-glucanase from ß-glucans in the samples are calibrated with the ß-glucan standard curve that is prepared with ß-glucan standards under the same conditions as for the samples. The ß-glucan content in the sample is then calculated.

B. Analytical Protocol

1. Enzyme Purification

a. ß-glucanase (Cellulase, EC 3,2,1,4; Catalog C-0901; Sigma Chemical Company, St. Louis, MO) derived from *Penicillium Funiculosu* is weighed (.5 g) directly into a 20-mL centrifuge tube and dissolved with 12 mL Buffer A (sodium acetate, .05 M, pH 4.0). Stir thoroughly for 10 seconds with a Vortex Genie™. The mixture is kept at room temperature for 10 min. Mix again for 10 seconds and centrifuge at 2,510 x *g* for 5 min.

b. The supernatant is transferred to screw-capped Pyrex tubes (4.0 mL per tube) and heated at 70° C for 60 min in a water bath, then plunged into ice water to terminate the heating process and left in cold water for 2 min. The heating time can be prolonged or shortened to such an extent that amyloglucosidase activity is completely destroyed, whereas ß-glucanase activity remains active and is able to hydrolyze up to 900 µg ß-glucans to glucose within 2 h at 40° C, or yield less than .2% glucose from 10 mg of starch within this time period.

c. Centrifuge again and transfer the supernatant to a Spectrapor membrane dialysing

tubing. The dialysing tubing is sealed at one end with a clamp and soaked beforehand in buffer A. The enzyme preparation (approximate every 40 mL) is dialysed against 4 L buffer A at 4° C for 16 h. The resultant solution is kept in storage at 4°C. The enzyme activity remains stable for a period of up to seven days at this temperature.

2. Pre-treatment of Samples (sample clean-up)

a. Samples are ground in a Wiley mill through a .3-mm sieve.

b. A series of screw-capped Pyrex test tubes (15 x 180 mm) are washed, ashed overnight at 450° C and identified. Samples are weighed out (.100 g) in triplicate directly into the test tubes.

c. Five mL 80% ethanol is added to each test tube and mixed thoroughly with a Vortex Genie™. The mixture is boiled gently in a water bath for 5 min to inactivate the endogenous α-amylase and amyloglucosidase. The test tubes are covered with glass marbles to prevent evaporation of ethanol.

d. Centrifuge at 2,510 x *g* for 5 min and discard the supernatant.

e. Add 5 mL 80% ethanol to each test tube and mix thoroughly with a Vortex Genie™ to wash the pellet. Then centrifuge at 2,510 x *g* for 5 min and again discard the supernatant.

f. Repeat step **e** one more time.

g. Re-suspend the washed pellet in 5.0 mL buffer B (sodium acetate, .05 M, pH 5.0) containing .02% (w/v) sodium azide as preservative. It is essential to store this suspension at room temperature. Do not chill before analysis, because a low temperature results in precipitation.

3. Standard Curve Preparation and Sample Hydrolysis

Standard curve preparation and sample hydrolysis are carried out in a same batch of assay, however, the steps of sample clean-up are not required for ß-glucan standards. To avoid confusion, the steps for standard curve preparation and sample hydrolysis

are described separately.

Standard Curve Preparation:

a. A series of 13 x 180 mm Pyrex test tubes are washed, ashed overnight at 450° C and identified (for example, STD1. ... STD10).

b. A stock solution of ß-glucan standards is prepared by dissolving 50 mg ß-glucan standard (barley ß-glucans, Sigma, Catalog G-6153) in 10.0 mL sodium acetate buffer B (5.0 mg/mL). The mixture is thoroughly mixed and heated at 90° C in a water bath for 5 min to keep it in uniform suspension. A series of dilutions are prepared from this suspension (Table A2-1) such that the ß-glucan contents in these standards cover the range of all samples to be analyzed (from previous knowledge or by trial and error). For example, It was reported that the ß-glucan content in barley ranged between 2 to 10% (Martin and Bamforth, 1981; Åman and Graham, 1987). Assuming that the ß-glucan content of the hulless barley is approximately 6.0% and the sample size is 100 mg, each sample will contain 6.0 mg ß-glucan (100 mg x 6.0%). Therefore, sample sizes containing ß-glucans from 0 to 12 mg will be appropriate to cover this range. In fact, observations from this study show that the linearity between absorbance at 520 nm and ß-glucan content deviates when the ß-glucan concentration is over 6.0 mg/tube.

Table A2-1. ß-glucan standard curve preparation

ID	Blk	Std1	Std2	Std3	Std4	Std5	Std6	Std7
Stock (mL)	0.0	0.1	0.3	0.5	1.0	1.5	2.0	2.5
Buffer B (mL)	5.0	4.9	4.7	4.5	4.0	3.5	3.0	2.5
ß-glucan (mg)	0.0	0.5	1.5	2.5	5.0	7.5	10.0	12.5
ß-glucanase (mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

c. One mL of purified ß-glucanase solution was added to each test tube. The amount of ß-glucanase used was greatly in excess of that required to hydrolyze all ß-glucans

in the solution during the 2-h incubation. Continuation of hydrolysis for 4 h did not increase the amount of reducing sugar produced (Henry, 1984).

d. When incubation is completed, the tubes are centrifuged at 2,510 x *g* for 5 min.

e. To verify whether the purified ß-glucanase is contaminated with α-amylase activity, amylose standards (Sigma, type III, lot 42H3861) were also included and treated exactly the same as the ß-glucan standards.

4. Enzymatic Quantification of Reducing Sugar

a. Prepare a series of 13x100 mm disposable borosilicate glass test tubes marked identical to the incubation tubes.

b. Reconstitute the Glucose Assay Reagent following instructions provided in the kit.

c. Reagent blanks: prepare two test tubes for reagent blanks to which .2 mL deionized water is added.

d. To the other tubes, .2 mL supernatant from the incubation tubes is transferred.

e. To all tubes, including reagent blanks, 1.0 mL reconstituted Glucose Assay Reagent is added.

f. The solution in the tubes is mixed by gently swirling and allow the mixture to stand for 5-10 min at room temperature (18-26 ° C) for the color to develop.

g. To each test tube 10.0 mL .1 M HCL is added and mixed with a Vortex Genie™.

h. The optical density of the reaction mixture is measured at 520 nm, against reagent blank as reference, with a Milton Roy Spectronic 3000 Array Spectrophotometer (Fisher Scientific, Edmonton, AB). **Standard Curve Determination** program is used to read the standards. Absorbance at 520 nm is plotted against contents (mg/tube) of ß-glucan standard and amylose standard (Figure A2-1).

Sample Hydrolysis for ß-glucan determination:

a. Pre-treated samples (from step 2) are hydrolyzed by purified ß-glucanase.

b. To each test tube containing samples (except for the third one in each triplicate, which is used as control), 1.0 mL purified ß-glucanase is added. To the control, 1.0

mL deionized water is added to replace the ß-glucanase.

c. Two enzyme blanks to measure the absorbance of ß-glucanase are included to which 5.0 mL buffer B and 1.00 mL purified ß-glucanase solution are added. The incubation, centrifugation and preparation for colorimetric reading were exactly the same as described for standard curve preparation.

The samples are then calibrated with the standard curve using the program **Unknown Sample Determination** or simply using the program **Absorbance / Transmittance** to read the absorbance against each individual sample blank as reference. The ß-glucan content in the sample is calculated using the following formula:

ß-glucans (mg/g sample) = [(A_{520} - .083)/.098]/sample wt (g).

Where A_{520} is the net absorbance of sample at 520 nm (A_{520} sample - A_{520} sample blank).

For example, if the average net absorbance of sample A (hulless barley) is .56 units and sample weight .127 g, then the content of ß-glucans in this sample will be 38.33 mg/g.

Note:

a. Complete readings with 30 min, because the optical densities of samples and standards decrease but that of the reagent blank increases with time.

b. If glucose content exceeds 30 mg/mL, dilute the mixture with .1 M HCL and repeat reading; the content of glucose is then calculated by multiplying the dilution factor.

c. A new ß-glucan standard curve must be prepared with each set of assay.

d. The maximum volume of one vial Glucose Assay Reagent is 21 mL after reconstitution which is sufficient for 20 assays. It is advisable to prepare a maximum of 40 test tubes in each set of assays (10 for ß-glucan standards, five for amylose standard

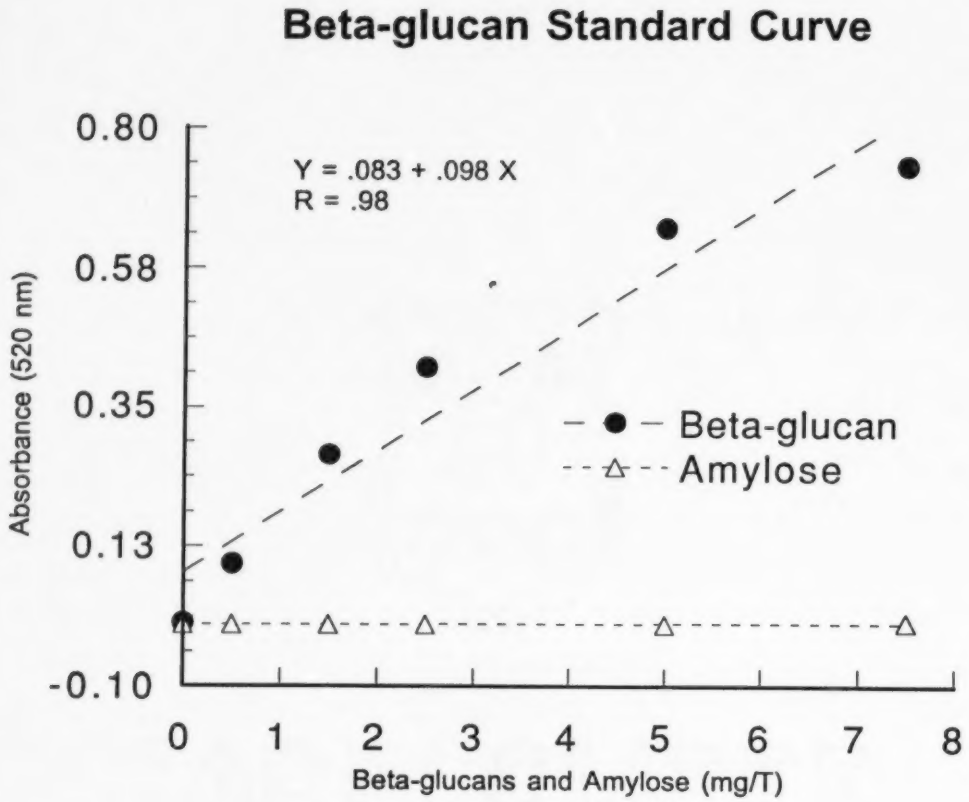

Figure A2-1.

Standard curve for ß-glucan determination. Absorbance at 520 nm (A_{520}) is plotted against content of ß-glucan standard (mg/tube, solid cycles) and amylose standard (mg/tube, triangles). The amylose standards are prepared in the same concentrations and under the same conditions as the barley ß-glucan standards.

C. Equipment And Reagents

1. Equipment:

a. High speed centrifuge (>3,000 x *g*)

b. Spectrophotometer with auto-sampling pump (Milton Roy Spectronic 3000 Array Spectrophotometer, Fisher Scientific, Edmonton, AB, Canada).

c. Shaking water bath capable of maintaining temperature at 40 ± 1.0° C and up to 100° C.

d. 20 mL centrifuge tubes.

e. Screw-capped Pyrex test tubes (15x180 mm) and disposable borosilicate glass tubes (13 x 100 mm).

f. Wiley mill with .3 mm screen (Arther H. Thomas Co., Philadelphia, PA).

g. Scale with .0001 gram accuracy.

h. Pipettes (200 µL, 1000 µL and 5.0 mL).

i. Vortex mixer (Vortex Genie™ , Scientific Industries, Bohemia, NY).

j. Spectrapor dialysing tubing (Spectrapor™ membrane tubing, MW cutoff 6,000-8,000, cylinder inside diameter 20.4 mm, Code 132655. Spectrum Medical Industries, Inc., 60916 Terminal Annex, Los Angles, CA).

2. Reagents:

a. ß-glucanase: Cellulase from *Penicillium Funiculosum* (Sigma, Catalog C-0901). Activity 5-10 U/mg solid. One U will liberate 1.0 µmol glucose from cellulose in 1 h at pH 5.0 and 37° C.

b. ß-glucan standard from barley (Sigma, Catalog G-6513).

c. Amylose standard from potatoes (Sigma, Type III, lot 42H3861).

d. Sodium acetate buffer A (.05 M, pH 4.0): dissolve 13.608 g sodium acetate (CH_3-$COONa.3H_2O$, FW = 136.08) in 1.5 L deionized water, adjust pH with 6 M HCL and make up the volume to 2 L with deionized water. Keep in storage at 2-6° C.

e. Sodium acetate buffer B (.05 M, pH 5.0): same procedure for preparing buffer A is followed. Sodium azide (NaN_3, FW = 65.01, Lot # 43H0291) is used as preservative and dissolved in the buffer at .02% (w/v). Caution should be exercised to avoid

inhalation or direct contact with skin or eyes of this chemical.

f. Hydrochloric acid (.1 M): 8.33 mL concentrated 12 M HCL is added to 500 mL deionized water and the final volume is adjusted to 1,000 mL.

g. 80% ethanol: 800 mL unhydrated ethanol is diluted with deionized water to 1,000 mL.

h. Glucose Procedure 115: (Sigma, Diagnostics™, Catalog 115-A).This reagent kit provides the following:

Glucose Color Reagent, Glucose Enzyme Reagent and Glucose Standard. The Glucose Assay Reagent is prepared by reconstituting one vial of Glucose Enzyme Reagent with 17 mL deionized water and 4.0 mL Glucose Color Reagent. Invert to mix, **do not shake**. This reagent is stable for 6 h at room temperature (18-26° C), 3 d in refrigerator (2-6° C) and at least one week when frozen.

Working principle:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK} + M_g^{++}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-P DH}} \text{6-PG} + \text{NADPH}$$

$$\text{NADPH} + \text{PMS} \longrightarrow \text{NADP} + \text{PMSH}$$

$$\text{PMSH} + \text{INT} \longrightarrow \text{PMS} + \text{INTH}$$

This analysis is based on the hexokinase (HK)-catalyzed conversion of glucose to glucose-6-phosphate (G-6-P). This reaction is coupled with the subsequent reduction of nicotinamine adenine dinucleotide phosphate (NADP) to NADPH by action of glucose-6-phosphate dehydrogenase (G-6-P DH). In the presence of NADPH, phenazine methosulfate (PMS) is reduced. The PMSH generated is then responsible for the reduction of iodonitrotetrazolium chloride (INT) to form INTH, which is measured colorimetrically at 520 nm. The colorimetric response is proportional to the glucose concentration.

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APPENDIX III

DETERMINATION OF SOYBEAN TRYPSIN INHIBITORS IN NUTRISOY

A. Introduction

The most widely used method for measuring activity of trypsin inhibitors (TI), which occur in soybean and other legumes, evolved from a procedure developed by Kunitz (1947) in which the hydrolysis of casein by trypsin was measured spectrophotometrically in the presence and absence of TI. When TI is present in the sample, trypsin is bound to TI to form an enzyme-inhibitor complex; thus the activity of the enzyme is partially or entirely inhibited depending on the amount of TI present in the sample. In subsequent studies, Erlanger et al. (1961) and Kakade et al. (1969, 1974) introduced a synthetic substrate, N_{α}-Benzoyl-DL-arginine-*P*-nitroanalide hydrochloride (BAPA), which reacts with the residual trypsin that is not complexed with TI. The end product, *p*-nitroaniline, produces a yellow color which shows maximum absorbance at 410 nm. With this synthetic substrate, the hydrolysis of substrate by the enzyme follows zero-order kinetics. A linear relationship between the quantity of *p*-nitroaniline released and the concentration of the active enzyme can be obtained within certain limits. Hamerstrand et al. (1981) and Kakade et al. (1969, 1974) reported a linear relationship between sample size and the differential absorbance (absorbance of trypsin standard minus absorbance of sample) within approximately 40-60% inhibition of the trypsin standard used. Below or above this range there will be a deviation from linearity, because a very small sample size will easily cause large variations; on the other hand, concentrations that are too high may result in disassociation of the enzyme-inhibitor complex (Green, 1953; Bundy and Mehl, 1958). Therefore, it is important to carry out preliminary tests to determine the amount of sample required to obtain 40-60% inhibition.

The standard AACC method (1969, 1983) for determining TI in soy products, using BAPA as substrate, produced data in the nonlinear portion of the absorbance versus sample size curve (over 60% inhibition). These values, when converted to trypsin inhibitor units per mL and extrapolated to zero concentration, tend to give erroneously high values of trypsin inhibitor content. To overcome these shortcomings, Hamerstrand et al. (1981) proposed a modified procedure that used only one sample dilution, instead of a series of dilutions, that produced a level of trypsin inhibition in the 40-60 60% range. Trypsin inhibitor content can be directly calculated. However, when this procedure was applied in our laboratory, there was no color reaction. Therefore, we changed the trypsin standard solution from .001 M HCL to Tris buffer (Tris [hydroxymethyl] aminomethane with calcium chloride, .05 M, pH 8.2) according to Tan and Wong (1982). However, in that and other reports there were no descriptions of procedures used to determine the appropriate sample size required to obtain 40-60% inhibition.

This procedure describes in detail the steps taken to estimate the sample size required to obtain 40-60% inhibition. In addition, based on principles of Hamerstrand et al. (1981) and Kakade et al. (1969) and using BAPA as substrate , a trypsin standard curve is prepared under the designed conditions. This standard curve shows a linear relationship between absorbance and trypsin concentration up to .45 absorbance units at 410 nm. In addition, an indirect estimation of the purity of the trypsin standard can be obtained according to this standard curve. This procedure was carried out to determine the total trypsin inhibitor activity in Nutrisoy (a food grade defatted soy flour, provided by Archer Daniels Midland Company, Decatur, IL).

B. Analytical Protocol

1. Sample Extraction

Finely ground and defatted Nutrisoy and autoclaved Nutrisoy are weighed out (1.0 g) into a 250 mL Erlenmeyer flask and extracted with 50 mL .01 M NaOH for 2-3 h (2 h for Nutrisoy and 3 h for autoclaved Nutrisoy). During extraction, the mixture is stirred

continuously to maintain a suspension. The pH is usually between 9.5 and 9.8; a higher concentration of NaOH should be used for extraction if the pH is lower than 8.4. This suspension is then diluted so that 2 mL extract can inhibit 40-60% of the trypsin standards used in the assay. The appropriate dilution is determined by a preliminary test described in the following section.

2. Preliminary Test to Estimate Sample Size Required to Obtain 40-60% Inhibition

Prepare a series of 13 x 100 mm Pyrex test tubes which are identified from 1 to 16 as shown in Table A3-1.

Table A3-1. Estimation ofthe quantity of samples required to obtain 40-60% inhibition of the trypsin standard

Tube #	Sample ID	Sample (mL)	Water (mL)	Trypsin Std (mL)	BAPA (mL)	30% acid (mL)	Total (mL)	Sample (mg)
1	Soy extract	0	2.0	2	5	1	10	.1
2	Soy extract	.1	1.9	2	5	1	10	.4
3	Soy extract	.3	1.7	2	5	1	10	1.2
4	Soy extract	.5	1.5	2	5	1	10	2.0
5	Soy extract	.7	1.4	2	5	1	10	2.8
6	Soy extract	.9	1.1	2	5	1	10	3.6
7	Soy extract	1.1	1.0	2	5	1	10	4.4
8	Soy extract	1.3	.7	2	5	1	10	5.2
9	Soy extract	1.5	.5	2	5	1	10	6.0
10	Soy extract	1.7	.3	2	5	1	10	6.8
11	Soy extract	1.9	.1	2	5	1	10	7.6
12	Soy extract	2.0	0	2	5	1	10	8.0
13	Trypsin Std	0	2.0	2	5	1	10	0
14	Trypsin Std	0	2.0	2	5	1	10	0
15	Reagent Blk	0	2.0	2	5	1	10	0
16	Reagent Blk	0	2.0	2	5	1	10	0

Take 2 mL of the initial extract suspension (concentration: 20 mg/mL) obtained from step 1 and dilute to 10 mL with .01 M NaOH (concentration: 4 mg/mL). Pipette into each test tube an increasing amount (from 0 to 2.0 mL) of the diluted extract suspensions and adjust volume with deionized water to 2.0 mL. If the preparation of the tubes takes a long time, a constant time interval between each tube should be arranged (e.g, 10 or 15 seconds).

After adding sample, water and trypsin, the tubes are incubated in a water bath at 37° C for 10 min with constant shaking.

Exactly 10 min after the initiation of incubation, 5.0 mL BAPA solution is added to each tube in the same order and time intervals between each two test tubes. Mix thoroughly with a Vortex Genie™ and incubate under the same conditions for another 10 min.

At the end of incubation, 1.0 mL of 30% acetic acid is blown into each test tube with a pipette to terminate the reaction. The acid is added in the same order and time intervals as the tubes are prepared for incubation,. For reagent blanks, acid is added **before** trypsin and BAPA.

Thoroughly mix the content and centrifuge at 2,510 x *g* for 10 min. The optical density of the supernatant is measured at 410 nm against reagent blank with a Milton Roy Spectronic 3000 Array Spectrophotometer with an auto-sampling pump (Fisher Scientific, Edmonton, AB, Canada). From the results obtained, it is easy to estimate the quantity of sample required for extraction.

Assuming that the average optical density of the trypsin standards is .45 absorbance units, an inhibition of 40-60% of this standard should give an absorbance around .18-.27 units. Select the test tubes that produced an absorbance within this range and then calculate the amount of sample required. For example, if the absorbance of tube #3 is .195, then there will be approximately 57% (1- .195/.45) inhibition of the standards. There is 1.2 mg sample in 2.0 mL mixture (.3 mL sample extract plus 1.7 mL deionized water). The amount of sample required to obtain this optical density will be:

1.2 mg/2.0 mL = .6 mg/mL or 60 mg Nutrisoy in 100 mL .01 M NaOH.

The final dilution factor is 1,667 (1 g sample is diluted so that 1 mL extract contained .6 mg).

3. Quantification of Soybean Trypsin Inhibitors in Nutrisoy Samples

Weigh 60 mg sample into an Erlenmeyer flask and fill with 100 mL .01 M NaOH for extraction as described for the preliminary tests. After extraction, 2.0 mL extract is pipetted, in triplicate, into 13 x 180 mm Pyrex test tubes as shown in Table A3-2.

Table A3-2. Preparation of samples for soybean trypsin inhibitor determination

Tube #	Tube ID	Sample (mL)	Trypsin (mL)	Water (mL)	BAPA (mL)	Acid (mL)	Trypsin (mL)	Total (mL)
1	Sample 1	2	2	0	5	1	0	10
2	Sample 1	2	2	0	5	1	0	10
3	Sample 1 Blk	2	0	0	5	1	2	10
4	Sample 2	2	2	0	5	1	0	10
5	Sample 2	2	2	0	5	1	0	10
6	Sample 2 Blk	2	0	0	5	1	2	10
7	Sample 3	2	2	0	5	1	0	10
8	Sample 3	2	2	0	5	1	0	10
9	Sample 3 Blk	2	0	0	5	1	2	10
10	etc.	2	2	0	5	1	0	10
11	Reagent Blk	0	2	2	5	1	0	10
12	Reagent Blk	0	2	2	5	1	0	10
13	Trypsin Std	0	2	2	5	1	0	10
14	Trypsin Std	0	2	2	5	1	0	10

|<---------- step 1 ----------> |<-step 2-> | <-step 3- > |<-step 4 -> |

Step 1. Sample extract, trypsin or water are incubated at 37° C for 10 min.

Step 2. Substrate (BAPA) is prewarmed to 37° C and blow rapidly, using a wide-tip pipette, 5.0 mL of this solution into each test tube, mix thoroughly and incubate for another 10 min at 37° C.

Step 3. The reaction is terminated by blowing 1.0 mL 30 % acetic acid into each test tube. In reagent blanks, the acid is added **before** the BAPA is added.

Step 4. The trypsin standard is added in the sample blanks **after** termination of the reaction.

Step 5. The mixture is mixed and centrifuged at 2,150 x *g* for 10 min, then the absorbance is measured at 410 nm.

4. Trypsin Standard Curve Preparation

Trypsin standard (Sigma, EC 3.4.21.4, type I, from bovine pancreas) is dissolved in Tris buffer (pH 8.2) and diluted so that 2 mL trypsin solution produces an absorbance that does not exceed .45 units at 410 nm. According to Hamerstrand et al. (1981) and Kakade et al. (1974), a stock solution is made by dissolving 18-20 mg trypsin standard in 100 mL Tris buffer (pH 8.2). The final solution is made by further dilution of 10 mL stock solution with the same Tris buffer to 100 mL (the final concentration is 18-20 μg/mL).

A series of test tubes are prepared and .1 to 2.0 mL diluted trypsin solution is pipetted into each test tube and volume adjusted to 4.0 mL with Tris buffer (pH 8.2). Five mL BAPA solution, which is prewarmed to 37° C, is added to each tube and incubated at 37°C for 10 min. The reaction is terminated by adding 1.0 mL 30% acetic acid and the optical density is measured spectrophotometrically at 410 nm against the reagent blank. A standard curve relating absorbance (410 nm) to trypsin content (μg/tube) is established as shown in Fig. A3-1.

The standard curve shows that a linear relationship between absorbance and trypsin content exists within the absorbance range of .00-.45 units with a slope of .009. From this relationship we can indirectly estimate the purity of the trypsin standard used. In this particular test, an increase of .009 absorbance units is obtained for each μg trypsin, which indicates that the trypsin standard used in this study has a purity of approximately 46% (.009/.019). This value is close to the purity of 56% reported by Kakade et al. (1969).

5. Calculations

The TI content in the sample is calculated based on the amount of trypsin units (TU) inhibited. One TU is arbitrarily defined as the increase of .01 absorbance units at 410 nm in a 10 mL mixture; the trypsin inhibitor activity is defined as the amounts of TU inhibited under the conditions defined (Kakade, et al., 1969).

To express trypsin inhibitor activity in terms of TU inhibited has the advantage that the activity of TI is independent of the purity of the trypsin used. However, since the purity of trypsin differs between various sources, so will the results from different laboratories.

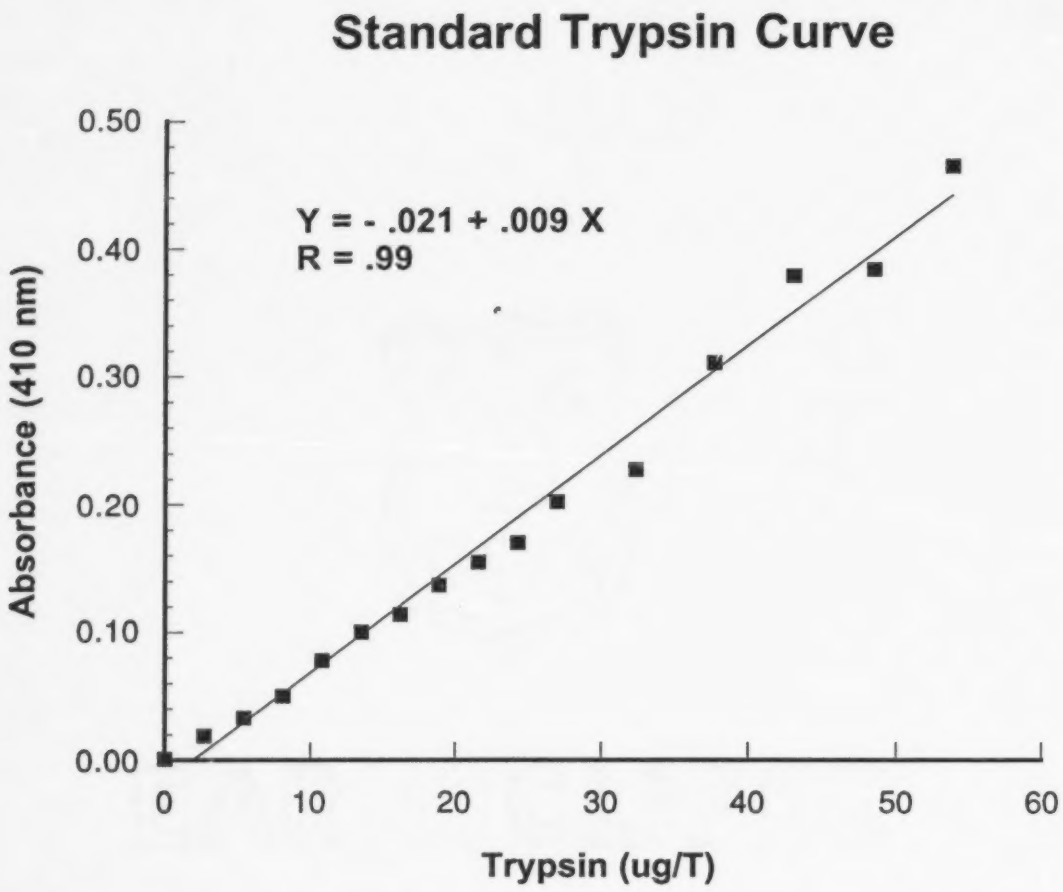

Fig. A3-1. Standard trypsin curve using N_{α}-Benzoyl-DL-arginine-*P*-nitroanalide hydrochloride (BAPA) as substrate. Curve is obtained by plotting absorbance (410 nm) against trypsin content (μg/tube).

For the purpose of comparison, it is desirable to express the inhibitory activity in terms of absolute units (e.g., μg) of pure trypsin inhibited. This can be accomplished by referring to the standard curve relating absorbance to trypsin concentration. Kakade et al. (1969), using purified trypsin, found that 1 μg "pure" trypsin has an activity of 1.9 TU which is equivalent to .019 absorbance units. For each μg pure trypsin inhibited, there will be a decrease of .019 absorbance units in the sample tested; similarly there will be an increase in these units if the differential absorbance between standard and sample is used.

Based on these findings, the TI content in the sample can be calculated using the following formula:

TI (mg/g) = ($A_{standard}$ - A_{sample})/.019 x (1mg/1,000μg) x dilution factor/sample (g)

Where $A_{standard}$ = Absorbance of trypsin standards,

A_{sample} = Absorbance of samples.

For example, in this particular test, when $A_{standard}$ = .45 and A_{sample} = .022 and if 60 mg sample is extracted in 100 mL NaOH (dilution factor 100), then the TI content in this sample will be:

TI (mg/g sample) = (.45-.022)/19 x 100/.06 = 37.5.

5. Chemicals and Reagents

a. Tris buffer (.05 M, pH 8.2):

Trizma® Base, (Tris [hydroxymethyl] aminomethane), ($C_4H_{11}NO_3$, FW = 121.1, Sigma, Catalog T-6791) 3.03 g and Calcium Chloride dihydrate ($C_aCl_2.2H_2O$, FM = 147.0, Sigma, Catalog C-7902) 3.68 g, are dissolved in 400 mL deionized water. The pH is adjusted to 8.2 with 1 M HCL and the final volume is made up to 500 mL with deionized water. This solution is prewarmed to 37° C for BAPA dilution. This buffer is stable for 30 h at room temperature.

b. N_α-Benzoyl-DL-arginine-*P*-nitroanalide hydrochloride (BAPA) (Sigma, Catalog B-4875):

BAPA (100 mg) is dissolved in 5 mL dimethyl sulfoxide (C_2H_4SO, FW = 78.13. Sigma, Catalog D-5857) and diluted to 200 mL with Tris buffer. Care should be

taken to ensure all BAPA is dissolved in dimethyl sulfoxide before further dilutions are made, because traces of crystals can cause precipitation. This solution is kept at 37° C during use; a fresh solution should be made for each assay.

c. Trypsin standard (Type II, from porcine pancreas, Sigma, Catalog T-7409):

A stock solution is made by dissolving 18 mg trypsin standard in 100 mL Tris buffer (pH 8.2). Take 10 mL of this stock solution and dilute with the same Tris buffer to 100 mL (the final concentration will be 18 µg/mL). The stock solution is stable for 7 d at 5° C; the diluted solution should be made fresh for each assay.

d. 30% acetic acid:

30 mL concentrated acetic acid (H_3H_2COOH) is added to 70 mL deionized water.

e. Hydrochloride acid (1.0 M):

83.3 mL 12 M HCL is added to deionized water and final volume adjusted to 1,000 mL.

f. Sodium hydroxide (.01 M):

Dissolve .4 g NaOH in 1,000 mL deionized water.

6. Equipment:

a. pH meter.

b. Pipettes (50-200 µL, 200-1000 µL and 1-5 mL).

c. Water bath with shaker (37-100° C).

d. Test tubes (13 x 100 mm) suitable for high speed centrifugation (over 2,000 x *g*).

e. Milton Roy Spectronic 3000 Array Spectrophotometer with auto-sampling pump (Fisher Scientific, Edmonton, AB, Canada)

f. Analytical balance, Vortex Genie™, hot plate, magnetic stirring bar, volumetric flask, funnels, etc.

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